THE JOURNAL OF NUTRITION

OFFICIAL ORGAN OF THE AMERICAN INSTITUTE OF NUTRITION

GEORGE R. COWGILL, *Editor*
Yale Nutrition Laboratory, 333 Cedar Street
New Haven 11, Conn.

EDITORIAL BOARD

WILLIAM J. DARBY
D. M. HEGSTED
PEARL P. SWANSON
VERNON H. CHELDELIN
CARL V. MOORE
HAROLD H. WILLIAMS
ALEX BLACK
FLOYD S. DAFT
HARRY G. DAY
JAMES B. ALLISON
CARL A. BAUMANN
LEO C. NORRIS

VOLUME 49

JANUARY – APRIL 1953

PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PENNSYLVANIA

CONTENTS

No. 1 JANUARY 1953

CLIVE M. MCCAY. Biography of Gustav B. von Bunge with frontispiece 3

LUTHER R. RICHARDSON, LYNN G. BLAYLOCK AND CARL M. LYMAN. Influence of the level of vitamins in the diet on the concentration of free amino acids in the plasma of chicks 21

I. P. AGRAWALA, C. W. DUNCAN AND C. F. HUFFMAN. A quantitative study of rumen synthesis in the bovine on natural and purified rations. I. Protein, dry matter and non-protein nitrogen 29

C. W. DUNCAN, I. P. AGRAWALA, C. F. HUFFMAN AND R. W. LUECKE. A quantitative study of rumen synthesis in the bovine on natural and purified rations. II. Amino acid content of mixed rumen proteins 41

AMOS E. LIGHT AND JOHN A. TORNABEN. Effects of prolonged percutaneous administration of methyl testosterone and estradiol on growing male rats. Ten figures 51

O. NEAL MILLER, J. W. GODDARD, ROBERT E. OLSON AND F. J. STARE. Folic acid deficiency in the duck. Ten figures 65

HIDEO NISHIMURA. Zinc deficiency in suckling mice deprived of colostrum. Eleven figures 79

H. NIÑO-HERRERA, M. SCHREIBER, R. A. COLLINS AND C. A. ELVEHJEM. Dermatosis in weanling rats fed lactose diets. II. Histological studies. Seven figures 99

DOUGLAS V. FROST, HOWARD H. FRICKE AND HENRY C. SPRUTH. Rat growth assay for vitamin B_{12}. Correspondence with collaborative microbiological assay results on U.S.P. liver extracts. Two figures 107

CHARLES D. MAY, AGNES HAMILTON AND CHARLES T. STEWART. Experimental megaloblastic anemia and scurvy in the monkey. V. Nature of the relation of ascorbic acid deficiency to the metabolism of folic acid compounds. One figure 121

K. R. JOHANSSON, G. E. PETERSON AND ELLIOT C. DICK. Effects of dietary aureomycin upon the intestinal microflora and the intestinal synthesis of vitamin B_{12} in the rat. Four figures 135

F. W. SHERWOOD AND VIRGINIA WELDON. Comparison of four feeding methods for assessing the relative growth-promoting properties of proteins 153

ARLENE P. MARTIN, CHARLES R. SHAW AND HAROLD H. WILLIAMS. Growth response of the rat to heterologous protein administered intraperitoneally. One figure 163

P. ROTHERY, J. M. BELL AND J. W. T. SPINKS. Cobalt and vitamin B_{12} in sheep. I. Distribution of radiocobalt in tissues and ingesta 173

G. P. LOFGREEN AND MAX KLEIBER. The metabolic fecal nitrogen excretion of the young calf and the true digestibility of casein. One figure 183

No. 2 FEBRUARY 1953

D. M. Hegsted. Protein, calorie and methionine intakes in relation to nitrogen balance. (A note on the paper by Mertz et al.) 191

Robert H. Udall and Clive M. McCay. The feed value of fresh bone 197

A. A. Kandutsch and C. A. Baumann. Factors affecting the stability of thiamine in a typical laboratory diet. One figure 209

A. E. Denton and C. A. Elvehjem. Enzymatic liberation of amino acids from different proteins 221

M. O. Schultze. Nutritional value of plant materials. VIII. Lactation failure of rats fed diets containing purified soybean proteins. One figure 231

M. O. Schultze. Nutritional value of plant materials. IX. The effect of various supplements on lactation failure of rats fed diets containing purified soybean proteins 245

A. E. Harper and M. C. Katayama. The influence of various carbohydrates on the utilization of low protein rations by the white rat. I. Comparison of sucrose and cornstarch in 9% casein rations 261

B. S. Schweigert and Barbara T. Guthneck. Utilization of amino acids from foods by the rat. I. Methods of testing for lysine. One figure 277

Barbara T. Guthneck, Barbara A. Bennett and B. S. Schweigert. Utilization of amino acids from foods by the rat. II. Lysine 289

John Haldi, Winfrey Wynn, James H. Shaw and Reidar F. Sognnaes. The relative cariogenicity of sucrose when ingested in the solid form and in solution by the albino rat 295

J. F. Elam, R. L. Jacobs, W. L. Tidwell, L. L. Gee and J. R. Couch. Possible mechanism involved in the growth-promoting responses obtained from antibiotics 307

Paul F. Fenton and Marian T. Dowling. Studies on obesity. I. Nutritional obesity in mice. Three figures 319

E. W. Crampton, R. H. Common, Florence A. Farmer, A. F. Wells and Donna Crawford. Studies to determine the nature of the damage to the nutritive value of some vegetable oils from heat treatment. III. The segregation of toxic and non-toxic material from the esters of heat-polymerized linseed oil by distillation and by urea adduct formation. One figure 333

Albert N. Booth, Robert H. Wilson and Floyd DeEds. Effects of prolonged ingestion of xylose on rats 347

Patricia T. McCarthy and Leopold R. Cerecedo. The vitamin A requirement of the mouse. One figure 357

The American Institute of Nutrition. The registry of nutritional pathology 369

No. 3 MARCH 1953

Benjamin H. Ershoff. Comparative effects of pantothenic acid deficiency and inanition on resistance to cold stress in the rat 373

Morton A. Schwartz and J. N. Williams, Jr. Effect of vitamins and natural products on liver ascorbic acid of sulfasuxidine-fed rats 387

THEODORE C. PANOS AND JOHN C. FINERTY. Effects of a fat-free diet on growing female rats, with special reference to the endocrine system. Eleven figures 397

DAVID R. WEIR. Leukocyte production in pantothenic acid-deficient mice. Four figures 425

R. F. SEWELL, J. K. LOOSLI, L. A. MAYNARD, HAROLD H. WILLIAMS AND BEN E. SHEFFY. The quantitative threonine requirement of the suckling pig. One figure 435

DIETER KOCH-WESER, J. DE LA HUERGA AND HANS POPPER. Effect of choline supplements on fatty metamorphosis and liver cell damage in choline and protein deficiency. Two figures 443

R. G. HANSEN, H. M. SCOTT, B. L. LARSON, T. S. NELSON AND P. KRICHEVSKY. Growth stimulation and growth inhibition of chicks fed forage and forage juice concentrate 453

W. W. WAINIO, BERTRAM EICHEL, HERBERT J. EICHEL, P. PERSON, FRANCES L. ESTES AND J. B. ALLISON. Oxidative enzymes of the liver in protein depletion. Eight figures 465

R. A. COLLINS, M. SCHREIBER, C. A. ELVEHJEM AND E. B. HART. Vitamin B_{12} and folic acid in milk diets 485

EDGAR A. HAWK AND C. A. ELVEHJEM. The effects of vitamin B_{12} and B_{12r} on growth, kidney hemorrhage, and liver fat in rats fed purified diets 495

R. BRAUDE AND B. CONNOR JOHNSON. Effect of aureomycin on nitrogen and water metabolism in growing pigs 505

MADELYN WOMACK, HARRIET A. HARLIN AND PEI-HSING LIN. The influence of the non-essential amino acids on the requirement of the adult rat for isoleucine, methionine and threonine 513

IRVIN E. LIENER. Soyin, a toxic protein from the soybean. I. Inhibition of rat growth 527

W. L. LEOSCHKE, R. J. LALOR AND C. A. ELVEHJEM. The vitamin B_{12} requirement of mink. Two figures 541

No. 4 APRIL 1953

LAWRENCE J. SCHROEDER, MICHAEL IACOBELLIS AND ARTHUR H. SMITH. Heat processing and the nutritive value of milk and milk products. Five figures 549

BACON F. CHOW, J. M. BURNETT, C. T. LING AND L. BARROWS. Effects of basal diets on the response of rats to certain dietary non-ionic surface-active agents 563

GERALD LITWACK, J. N. WILLIAMS, JR., PREMA FATTERPAKER, LILLIAN CHEN AND C. A. ELVEHJEM. Further studies relating liver xanthine oxidase to quality of dietary protein 579

R. A. COLLINS, M. SCHREIBER AND C. A. ELVEHJEM. The influence of relative humidity upon vitamin deficiencies in rats. One figure 589

BRUCE KENNELLY AND LEONARD A. MAYNARD. The interrelationship between thyroid activity, dietary fat and lipogenesis 599

IRVIN E. LIENER AND ELDON G. HILL. The effect of heat treatment on the nutritive value and hemagglutinating activity of soybean oil meal. One figure 609

LOUIS J. PECORA. Effect of antibiotics on rats fed amino acid-supplemented rice diets 621

I. P. AGRAWALA, C. F. HUFFMAN, R. W. LUECKE AND C. W. DUNCAN. A quantitative study of rumen synthesis in the bovine on natural and purified rations. III. Riboflavin, pantothenic acid and niacin 631

ROMAN KULWICH, LEE STRUGLIA AND P. B. PEARSON. The effect of coprophagy on the excretion of B vitamins by the rabbit 639

J. GUZMAN-GARCIA, W. B. SARLES AND C. A. BAUMANN. Microorganisms in the intestines of rats fed penicillin 647

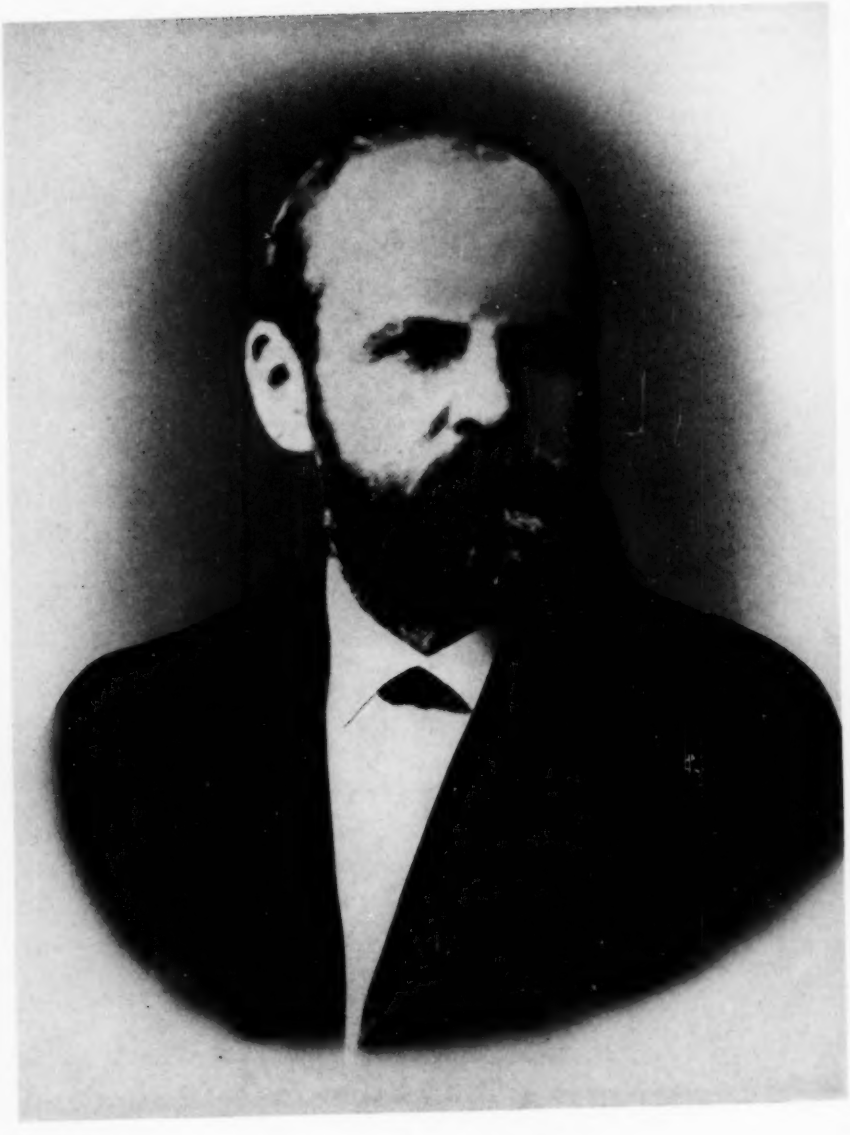
BACON F. CHOW, ROBERT L. DAVIS AND SHANLEY DAVIS. The effect of antibiotics and the composition of diets on fecal vitamin B_{12} and biotin 657

ROBERT A. HARTE, BACON F. CHOW AND LOIS BARROWS. Storage and elimination of vitamin B_{12} in the rat. One figure 669

CARL M. LYMAN, WAN YUIN CHANG AND J. R. COUCH. Evaluation of protein quality in cottonseed meals by chick growth and by a chemical index method 679

GUSTAV B. von BUNGE

(1844 – 1920)



GUSTAV B. von BUNGE

GUSTAV B. von BUNGE

(January 19, 1844 — November 5, 1920)

Gustav B. von Bunge was born in Dorpat, Esthonia, in 1844. He lived in Dorpat all of his early life and completed his degree in chemistry at the local university in 1874. In 1882 he received his medical degree in Leipzig. In 1885 he became professor of physiology in Basel, Switzerland. He died in 1920.

The father of Gustav von Bunge was a famous botanist, Alexander von Bunge. He was born in Kiev, but was educated at Dorpat where he passed through the gymnasium and also took his medical degree during the period of 1821-1825. Alexander wandered far afield in his botanical studies. In the course of these investigations, he spent many months in Siberia and China. During part of this period he accompanied the naturalist A. von Humboldt. After serving as botany professor at Kazan in 1835 von Bunge's father returned to Dorpat in 1836 where he taught until 1867. He continued to live in Dorpat until 1881 during which time he made many important contributions to the plant life of Esthonia. During this period Dorpat became so important in plant science that it was regarded as the center of this field for the whole of Russia.

Since both Alexander and his son Gustav were closely associated with the University of Dorpat during much of the 19th century when this institution grew from a small unknown school to an outstanding university, this school deserves attention because it provided both the major part of the education and research facilities for both father and son.

The small city of Dorpat is located in Esthonia on one corner of a triangle connecting Helsinki, Finland, and Leningrad, Russia. Dorpat is about 150 miles from each of these

Copyright 1953
The Wistar Institute of Anatomy and Biology
All rights reserved

well-known cities. On modern maps the name Dorpat has been replaced by the ancient Esthonian name of Tartu.

Although the University was founded in 1632 by the Swedish ruler, Gustavus Adolphus, it did not begin to rise to eminence until it was reopened by the Russian Tsar Alexander in 1802. Although Esthonia was a part of Russia during the whole of the 19th century, the University was almost entirely German. The staff was German and the language spoken was German until about the period of 1880–1887 during which the institution was Russianized and this language was made the official one.

This University was unique inasmuch as it was run by and for the minority of Germans living in Esthonia, the surrounding Baltic states, and Russia. During much of the 19th century the majority group, the native Esthonians, were not often admitted to the University. Thus we observe a minority group operating its own university and keeping out the majority. Even as late as 1922, there were living in Dorpat only 3,400 Germans and 54,000 Esthonians.

One of the most critical descriptions of the University was written by the father of modern embryology, Karl Ernst von Baer (1792–1865) in his autobiography published in 1865. During the time when Napoleon was retreating from defeat in Russia (1810–1814), von Baer was a student in the reorganized University of Dorpat.

Von Baer describes the difficulties of teaching science and medicine in a small community such as Dorpat. Limited clinical material was available and it was even difficult to get enough bodies for dissection in the anatomy course.

Furthermore, the young University had great difficulty in hiring professors from outside Esthonia because the Napoleonic Wars had destroyed gold and currency exchange. Hence the young University was staffed mostly by physicians whose university training had taken place 20 years or more before they started teaching.

However, even in this early period von Baer received thorough training in physics by G. Fr. Parrot. His life work

was inspired by his natural history teacher, Ledebour, who was really a botanist and taught zoology as a duty. Like many a modern student von Baer was turned against chemistry, forever, due to a poor teacher. The anatomy teacher of those times was K. F. Burdach, a textbook writer of note and an able teacher who seems to have owned no microscope in von Baer's day.

To most nutritionists the name of the University of Dorpat has a permanent place in history because in the year 1852 the famous work on metabolism by F. Bidder (1810–1894) and Carl Schmidt (1822–1894) appeared. This appeared in only one edition in Leipzig, under the title of "Verdauungssaefte und der Stoffwechsel." Today this excellent work of 413 pages still provides a useful source of information for serious students of metabolism who are willing to labor without benefit of an index.

Both Bidder and Schmidt joined the staff of Dorpat at about the same time as the elder von Bunge. Friedrich Bidder, as described in Lusk's book on nutrition, was born in Livonia, Russia and graduated in medicine at Dorpat in 1834. He returned to Dorpat after a year of further physiology training. He taught at Dorpat for 34 years and his pupils produced 77 doctors' dissertations. Carl Schmidt was born in Mitau, Courland, Russia. After taking his degree under Liebig in 1844 he returned to Dorpat and taught there for the next 45 years.

In several places the younger Gustav von Bunge refers to this famous team of a physiologist and a biochemist as his much respected teachers. Early in his professional life von Bunge acquired his interest in the inorganic constituents of the animal body. This interest was natural for the students of Carl Schmidt since as early as 1850 Schmidt was analyzing blood for potassium, sodium, phosphorus and calcium. At this time Schmidt found that most of the potassium was in the cells while the sodium was in the plasma.

In the field of pharmacology at Dorpat there was also early interest in potassium because Rudolf Buchheim (1820–

1879) was professor at Dorpat (1846–1867). He investigated the effect of potassium salts upon the animal body.

During von Bunge's student days the well-known pharmacologist, Oswald Schmiedeberg (1838–1921), who followed in the footsteps of Buchheim, extended the knowledge of potassium salts. Schmiedeberg and Buchheim were joint authors of a number of papers in pharmacology during the period 1853–1873 concerning the action of potassium iodide and other potassium salts.

The studies of the youthful von Bunge before he received his degree in chemistry at the age of 30 are worthy of careful consideration because they laid the background for his interests during the remainder of a long and active life of teaching and research. During this period he became especially interested in sodium chloride and its metabolism. He believed that this was the only inorganic nutrient of primary importance to the body when it is eaten in the form of a salt. He felt that other inorganic substances such as iron and calcium were utilized by the animal body chiefly as organic compounds found in foodstuffs.

In 1874 von Bunge published one of the most interesting papers ever written upon sodium and potassium chloride. This paper makes evident his extensive knowledge and interest in the food habits of peoples scattered over the face of the earth. He was very conscious of the important part salt plays in the culture and food habits of various races of men. Much of this knowledge and interest must have been derived from the experience and information gained by his father. During this period von Bunge was already much interested in the need for salt by vegetarians and the disregard of salt as food by meat eaters among both men and animals.

At this time von Bunge had started work on a subject that held his interest the rest of his life, namely, the interplay within the body of potassium and sodium. He had noted that potato eaters consuming as much as 20 to 40 gm of potassium in their diet could not eat potatoes for long periods unless they had liberal amounts of sodium chloride. He related such

observations upon man to animal research involving feeding or withholding salt from farm animals. At the time von Bunge was born (1844) Boussingault had already shown that farm animals decrease their food intake considerably if the diets lack salt. Von Bunge was familiar with this earlier research, since he was a very careful student of the literature from his earliest years.

As early as 1871, when 27 years old, von Bunge was writing about the merits of rice as a food because it placed so little burden on the kidneys. He recognized its value as the major component for low salt diets. Von Bunge appreciated the problem of the kidneys in the human body in comparison with such organs as the stomach. If diets proved unsatisfactory, the stomach had the power to rebel and to eject unsuitable food mixtures. However, the kidneys had to deal with all materials forced upon them from the blood stream. In his texts von Bunge stressed the fact that the kidneys were often injured beyond recovery before there was any recognition of damage.

During his student days, prior to 1874, von Bunge also became interested in the composition of both blood and milk. He recognized that milk was deficient in iron and began his speculations upon how the young animal fed solely upon milk could provide iron for the synthesis of hemoglobin. During this period von Bunge also extended the studies of his teacher, Schmidt, upon the inorganic constituents of blood and plasma. This interest in blood was to continue throughout life and terminate in the extensive analyses made by von Bunge's student, Adberhalden. These latter analyses, made more than 50 years ago, are still commonly reproduced in textbooks.

This interest in the composition of milk led to the attempts of von Bunge's student, Lunin, to maintain mice upon synthetic milk. The results of these studies appeared in Lunin's thesis published at Dorpat in 1880, and in the Zeit. Physiol. Chem., in 1881. Lunin disappears from the scientific stage after this one brilliant appearance, but through his work

von Bunge was convinced that milk contained organic essentials that the animal body could not make. He believed that these might be special organic compounds of cations such as iron. This working hypothesis was strengthened as the years passed and he became fascinated by the various forms of iron found in hemoglobin, liver and eggs.

Von Bunge's interest in the metabolism of iron was further stimulated by his familiarity with the early studies upon the metabolism of cats, published by Bidder and Schmidt, when von Bunge was only 6 years old. These teachers of von Bunge had found 6 to 8 times as much iron in the feces of fasting cats as they found in the urine. Hence they concluded iron must be excreted for the most part by way of the intestine rather than through the urine.

While von Bunge and Lunin were laboring at Dorpat, contemporary scientists in other institutions were also attempting to determine why animals could not live upon diets that were supposed to be complete according to the analyses of the chemists. Thus in 1869, J. Forster started attempts to feed dogs and pigeons upon synthetic diets with and without salt. These led to the famous report of 1873 in which Forster described polyneuritis in the dog but ascribed this condition to lack of salt in the diet. Unfortunately, Forster did not attempt to cure the condition by adding salt to the diet of the dog, and left this obvious test to be run by later critics of his studies.

Problems concerning the relationship between dietary iron and blood formation were attracting increasing interest in several laboratories during the formative years in von Bunge's career. As early as 1849 Verdeil found that dogs fed meat had more iron in their blood than those restricted to a diet of bread and potatoes. Preyer made extensive analyses upon the blood of many species and published his values in 1866. He accepted the erroneous value of 0.42% for the amount of iron in hemoglobin. This value of Carl Schmidt was later to be corrected by pupils of his student, von Bunge, to that now accepted, namely 0.34%.

While von Bunge was a student the debate was already increasing over the relative merits of inorganic salts of iron and organic iron compounds in their utilization by the body. Thus, Quincke's well-known paper upon the fate of iron salts appeared in 1868, and the extensive report of the effect of food upon the hemoglobin content of blood by the Russian, V. A. Subbotin of Kiev, was published in 1871. In 1878 appeared the studies of iron balances upon dogs fed various diets by E. W. Hamburger. Studies upon the iron content of bile were also made by Hamburger, but he could find no evidence of increase in the iron content after the feeding of iron salts so he added doubt to the question of the absorption of inorganic iron and added strength to von Bunge's theory that iron must be present in the food in organic combination if it is to be utilized by the body.

During his early years as a chemist von Bunge undertook various ventures in diverse fields. Thus, in 1873 he published in the Proceedings of the Natural History Society of Dorpat (vol. VII) an article purporting to show that urea of the urine was tied in some manner to uric acid. He must have felt this work was sound, since it was discussed in his text-books 30 years later.

In 1873 von Bunge also shared in the widespread interest in the nutritive value of Liebig's beef extract. He concluded that it had no special merits except that it tasted and smelled good to man. Others had used rabbits and injected this extract, but von Bunge concluded the rabbit was not a satisfactory animal for such studies since it so readily responded by changes in pulse rate to so many influences. At this time von Bunge was also pondering over the problem of why the injection of small amounts of potassium chloride into the blood stream would stop the heart, while a man eating potatoes could ingest as much as 50 gm of potassium without apparent injury.

During his early years von Bunge worked with Schmiedeberg for a time upon the problem of the site of formation of hippuric acid. In 1876 they reported that frogs could effect

this synthesis after removal of the liver, but dogs could not do so if the kidneys were removed. Hence, they concluded, the site of formation was in the kidneys.

The problem of intestinal putrefaction also engaged the attention of young von Bunge. As early as 1874 he noted that increased intestinal putrefaction led to increased sulfate sulfur in the urine. He was probably the first to make this observation.

During the decade starting in 1880 with the publication of Lunin's thesis, the interest both in the metabolism of iron and the use of purified, synthetic diets increased. Thus in 1882 H. V. Hoesslin fed dogs diets composed of egg white, starch, lard and a salt mixture modeled after the ash of milk. Some of the dogs were given cream from milk. The technique of using glass cages was employed. The value of iron in blood regeneration was studied by removing large amounts of blood from the jugular. These procedures of von Hoesslin, published 75 years ago, are widely employed today.

In 1883 as the result of the injection of iron into rabbits, Glaevecke concluded that this iron was deposited in the liver as an iron protein compound. During this same year also appeared the extensive review of iron and manganese metabolism by R. Kobert, who was born in Dorpat but not trained in that University.

During this period von Bunge completed his medical degree at Leipzig (1882) and started his arduous attempts to isolate the key organic iron compound of eggs. In 1884 and 1885 von Bunge reported his isolation of "hematogen" from the yolk of eggs. He believed this was the precursor of hemoglobin in the chick. In addition to iron his compound was rich in phosphorus and contained traces of Ca, Mg and Cl. He presented a table comparing the composition of his compound with nuclein and hemoglobin. In this table he still used the old value of 0.42 for the iron content of hemoglobin; his student Zinoffsky established the true value of 0.34% at about this date.

During this period von Bunge pursued some of his earlier interests and published the first edition of his small text on vegetarianism in 1885. During this time he also became interested in the chemistry of muscle contraction and completed a series of studies upon the round worms of cats. These were published in 1883, 1888 and 1889. Since this ascarid could live for long periods without oxygen, von Bunge concluded that muscles must be able to contract without oxygen.

While attempting his isolation of iron compounds from egg yolk, von Bunge was already familiar with the work of Miescher (1844–1895) in the isolation of compounds from the nucleus of salmon eggs. Von Bunge and Miescher were born in the same year, 1844. Both were the sons of famous university professors. Miescher's father (1811–1886) devoted most of his life to the teaching of anatomy and histology at Basel, while the elder von Bunge was a contemporary at Dorpat.

The younger von Bunge joined the staff as professor at Basel in 1885, so the labors of the younger von Bunge and Miescher took place in the same city until Miescher died in 1895. Although von Bunge studied the eggs of chickens and Miescher those of salmon, there is no evidence of cooperative work. However, von Bunge must have had high regard for the work of Miescher, to whom he refers in his texts.

While von Bunge was isolating iron compounds from egg yolk, other workers were attempting similar isolations from liver. Thus in 1885 S. S. Zaleski claimed the isolation of a substance he called "hepatin." The Zaleski papers concerning the iron in liver directed von Bunge's interest toward the importance of the liver in providing a reservoir of iron during the growing period when the young mammal has no food other than milk.

Starting in 1889 von Bunge published a series of papers in the Zeit. Physiol. Chem. dealing with the relation of this store of iron to the synthesis of blood in the animal organism. During this period he analyzed the bodies of rabbits, puppies, guinea pigs and cats of different ages. Rabbits of 16 different

ages from one hour to 74 days were analyzed. Likewise, he analyzed guinea pigs from an age of 6 hours to 53 days, puppies from 10 hours to 4 days and kittens from 4 to 19 days. He also analyzed the milk of dogs.

From these analyses von Bunge concluded that animals such as rabbits or dogs must have a liberal store of iron in their bodies at birth in order to make survival possible until they obtain iron in their food at the time of weaning. In contrast he concluded that the guinea pig had little reserve of iron in its body at birth because it did not need it. This species starts eating iron-rich food soon after birth.

During this same period F. Kruger (1890) reported an extensive study of the iron content of liver in relation to the age of the animal. He found the iron content of the liver of the foetus to be 10 times as rich as that of the adult. He also found the foetal spleen was not the storehouse for iron. Some of Kruger's results have never been checked, such as the claim that the spleen of the ox has only a 5th as much iron as that of the cow.

In 1890 appeared the analyses of hemoglobin by A. Jaquet of von Bunge's laboratory. He prepared and analyzed crystalline hemoglobin from dog and chicken blood and compared them with the earlier analyses of Zinoffsky. These values are interesting today and were as follows:

	Horse	Dog	Hen
	%	%	%
C	51.1	54.6	52.5
H	6.7	7.2	7.2
N	17.9	16.4	16.4
S	0.39	0.57	0.86
Fe	0.335	0.336	0.335

Thus the iron values were established which provide one of the modern methods for the determination of hemoglobin in blood.

Likewise, long consideration of the composition of blood made von Bunge very aware of the wastage of human food when blood from animals was discarded. In his texts he devotes careful consideration to this problem of wasted blood

and notes the use of blood in some areas for the preparation of sausages. Today this problem remains unsolved in America, where a vast tonnage of blood proteins is wasted each year by being dried into tankage and used for animal rather than human food.

The excellent analyses of hemoglobin were not the only ones published from von Bunge's laboratory in 1890. During that same year appeared the work of C. A. Socin concerning the form in which iron is utilized. Socin went to great trouble in preparing iron-low diets to feed to mice. He decided that even egg albumen had too much iron to serve as a protein source for such experimental diets. Hence, he used horse blood serum. For fat he tried to prepare iron-free butter, but found lard to be much easier to purify as Forster had learned 20 years earlier. He followed the practice of earlier workers in preparing a salt mixture similar to the ash of milk and mixing it into the diet in the ratio that the ash has to the nitrogen of milk. He even included a small amount of cellulose in his rations.

Unfortunately for his mice but luckily for science, Socin failed to add any sources of vitamins to his experimental diets. His mice died in the course of about a month in spite of feeding supplements of iron chloride, or "hematogen," or hemoglobin. The control animals fed egg yolk of course received vitamins; these mice survived so well that some had to be killed in order to end the experiment.

In the course of this study Socin fed not only mice but some dogs. The dogs were not given "synthetic" diets but such large amounts of egg yolks that they developed severe diarrhea. This study of dogs yielded little fruitful data but in the course of it Socin did find that the yolks of eggs varied substantially in their content of iron at different seasons of the year.

In concluding his report Socin asked himself certain questions about the nature of the unknown essential found in egg yolk, "Was it fat-like?", "Was it a lecithin compound?", "Must all inorganic compounds be combined in special organic structures?", and "Are proteins unequal in value and are

there certain fractions that are absolutely essential?'' Such were the perplexing problems 60 years ago.

In the nineties of the past century many papers appeared concerning the much debated problem of whether or not the body can assimilate inorganic iron compounds. These studies contributed to modern knowledge because experience was gained in feeding special diets to test animals. Most studies were made with mice and dogs, but the rat was also introduced. In the course of developing better methods for studying iron assimilation, von Bunge remembered his early analyses of foods such as milk and rice that were poor in iron.

In 1892 von Bunge introduced the technique of feeding milk and rice diets to produce anemia in mice without killing the animals. Hausermann used this technique in feeding rats in von Bunge's laboratory and reported his results in 1897. He made comparable studies with dogs, rabbits and cats. He found that rabbits would not tolerate the rice-milk diet so he fed them, as well as the dogs, only milk. Hausermann's cats became quite anemic when fed only milk. This species also developed diarrhea but recovered from both the anemia and this condition when fed meat. Even today comparatively little is known about the nutrition of the cat.

In 1897 Hausermann also published extensive tables of the iron values of foods. He used the analytical method for iron devised by von Bunge.

During this period of the nineties many attempts were made to prepare and test various organic compounds of iron. In the laboratory of Gaule, special feeding trials with mice were made by W. S. Hall to test ''carniferrin.'' In the same year (1894) as Hall's report appeared that of Schmiedeberg, von Bunge's associate at Dorpat. Schmiedeberg made a very extensive study of the value of ''ferratin'' and studied various synthetic iron-albumin compounds.

The stimulus of work from von Bunge's laboratory led others to attempt to improve ''synthetic'' diets. Thus in 1896 the diets of W. S. Hall were made of purified casein, starch, butterfat, cellulose and mixtures of inorganic salts.

Various salt mixtures were tried but the mice always died. The climate for the discovery of the vitamins was improving, however. In one of the reports of that year Hall devoted an entire report to the preparation of synthetic diets. Consideration was even given to the acid-base balance of the inorganic constituents.

During this period substantial opposition arose to von Bunge's theory of the essential nature of organic iron. A number of studies were made feeding manganese with and without iron. Several supporters of von Bunge had advanced the theory that inorganic iron was not utilized directly but did tie up sulfides in the intestinal tract, and thus made the wastage of organic iron much less. Workers like Woltering (1895) found that manganese tied up sulfide compounds but did not affect iron storage. Hence he concluded the theories of von Bunge were discredited.

Near the close of the past century two of von Bunge's students arose to his defense. They were Hausermann and Emil Abderhalden; the latter was destined to become famous and also to write the obituary of his master, von Bunge, in 1920.

Anticipating his large scale experiments of the future, Abderhalden used 199 rats, 66 rabbits, 72 guinea pigs, 21 dogs, and 23 cats for the study of the absorption of various forms of iron. He concluded that hematin made from blood provided utilizable iron but that inorganic compounds did not. During 1898–1899 he also concluded that rice bran and wheat bran are very good sources of utilizable iron.

These results stimulated von Bunge in his texts to write favorably about breads made from whole grains. He cites 4 reasons for his preference for whole grain breads over white breads, namely, (1) higher iron content, (2) higher calcium content, (3) the regulatory effect of cellulose on the intestine, and (4) the cleansing action of whole cereals upon the teeth.

As the century closed, we find that Abderhalden not only took over the torch for organic compounds of iron but also

extended von Bunge's work in regard to the composition of blood and milk. For many years von Bunge had believed that the concentration of the milk ingredients was directly related to the rate at which the young of a given species were obliged to grow. Abderhalden extended the analyses upon which this theory was based.

At the close of the century in 1899 von Bunge returned to his early interest in salt and published a study upon the sodium chloride in bones. He found that the salt content of bones dropped as a young animal develops. This work seems never to have been repeated.

At the turn of the century we find von Bunge taking much interest in newer aspects of human nutrition. He became interested in problems of acid-base balance in relation to the composition of the diet. He compared the composition of the urine after 24 hours upon a bread diet with that after a similar period upon pork. He noted the effects of the sulfur of meat proteins in helping to make the urine acid and of the alkaline salts of organic acids of fruits and vegetables in producing alkaline urine. He also noted this effect of eating potatoes.

From his laboratory in the period of 1898–1901 appeared a series of reports indicating that fruits decrease the excretion of uric acid and hence a fruit diet is useful in the treatment of gout. These reports seem to have been based upon the studies of his student, Weise.

The several editions of the textbooks of von Bunge afford interesting reading in either the original German or in English translation. The second edition of his textbook of Physiological and Pathological Chemistry appeared in translation by L. C. Woolridge in 1890. This work appeared in 1891 in a French translation by A. Jaquet, who had established the value of iron in hemoglobin. Finally this work was transformed into the second volume of the Lehrbuch der Physiologie des Menschen. This passed through two editions in 1901 and 1905.

One of von Bunge's most popular works was a small booklet of 32 pages first published in Munich in 1900. This dealt with the increasing inability of women to nurse their infants. It passed through 4 editions with the last in 1908. It was translated into French.

The booklet upon vegetarianism passed through two editions. The one upon alcoholic poisoning and degeneration passed through three German editions between 1904 and 1910. This also appeared in an English translation.

The textbook upon organic chemistry entitled Lehrbuch der organischen Chemie fur Mediciner in Siebzehn Vortragen passed through two German editions in 1906 and 1913. It was translated into English by R. H. A. Plimmer in 1907.

Two of von Bunge's works written late in life are little known. These are Die Quellen der Degeneration (1910) and Die Ausrottung der Geschlechts Krankheiten (1911).

The German texts of von Bunge are fairly common in old book shops, but the English translations were probably issued in small editions and are relatively rare. Every student can profit from owning one of these editions because von Bunge was an excellent writer and well grounded in the literature of both biochemistry and physiology. His outlook was that of a modern nutritionist.

From these texts one obtains many sidelights upon his researches and theories. Early in his career he concluded that the reason a herbivorous animal needs sodium chloride in its diet is the high content of potassium in plant materials. This potassium causes the sodium to be lost from the blood and urine and hence salt is needed to re-establish the equilibrium. Some of the early studies in testing this thesis were made upon von Bunge himself who determined sodium and potassium balances upon himself.

In considering salt substitutes von Bunge was aware that some tribes in Africa used the ashes of certain plants in lieu of sodium chloride. He had some of these plants shipped to him and found that certain plant ashes were rich in sodium.

Von Bunge believed that most people add too much salt to their food and thus put an extra burden upon the kidneys. He believed that one or two grams of salt daily are adequate for most people although some consume 20 or 30 gm daily.

Some of the worries of von Bunge over the diets of 50 years ago sound quite modern. Thus he felt infants were much better and had a better chance of survival when nursed by the mother. He believed the diets of most women were too low in calcium during the period of gestation and lactation.

The greatly increased use of sugar during his time also worried von Bunge. He noted the increase from 14 kg per person per year in 1851 to 36 kg in 1890. Perhaps he turns in his grave as he observes the still greater use today. He realized it was a cheap form of energy but was frightened because it displaced natural foods that carried other ingredients.

In later years von Bunge also became interested in the problem of alcoholism and wrote upon this subject. He even believed that problems of rickets in children, caries of the teeth and failure to secrete milk by women might be related to alcoholism. Today we realize these relationships may well have been true because these troubles are related to poverty in diets and wastage of family resources so that good foods cannot be purchased when funds of poorer families are spent for liquor.

Although von Bunge appreciated the virtues of milk as food, he felt that the diet of the infant should be supplemented by other foods quite early. He advocated a teaspoonful of spinach when the child had 8 teeth. This seems to have been the recommendation of one of his friends practicing pediatrics. He felt that nursing too long without other food produced anemia in the infant. He records the case of one student in Basel who had lived for 18 years upon little except milk. This student was only mildly anemic, however.

The labors of von Bunge and his students advanced our knowledge of mineral metabolism and helped lay the foundations for the discovery of the vitamins. They provided work-

ing techniques with "synthetic" diets and methods for the study of anemia using rice and milk diets. The texts of von Bunge are so well written that they have lasting value in providing historical and background knowledge in nutrition.

In the obituary to von Bunge by Abderhalden, his great interest in decreasing the use of alcohol is stressed. He believed alcoholism was one of the great reasons for human poverty and disease. He also believed smoking was partly responsible for many diseases of later life. In the obituary (Schweiz. Med. Wochn., *50:* 1192) the enthusiasm of von Bunge for prohibition in the United States is described. Fortunately, he did not live to see the end of this experiment, since he died of lung disease in 1920.

CLIVE M. MCCAY

INFLUENCE OF THE LEVEL OF VITAMINS IN THE DIET ON THE CONCENTRATION OF FREE AMINO ACIDS IN THE PLASMA OF CHICKS

LUTHER R. RICHARDSON, LYNN G. BLAYLOCK [1] AND CARL M. LYMAN
Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas Agricultural and Mechanical College System, College Station

(Received for publication August 5, 1952)

INTRODUCTION

A number of investigators have demonstrated that certain vitamins, amino acids and antibiotics have a sparing effect on each other for growth, and it seemed possible that they might also affect the free amino acid concentration of the blood. In this case some insight into the biochemical mechanisms of the interrelationship of different nutrients might be obtained by a study of their effect on the free amino acids of the plasma. In order to test the possibility of using this method of approach to investigate interrelationships of nutrients, a study was made with chicks to determine the influence of an adequate and a low level of pyridoxine, pantothenic acid, riboflavin, choline, niacin and vitamin B_{12} on the free arginine, lysine, methionine, tryptophan and valine contents of the blood plasma. The results of this study are described in this report.

EXPERIMENTAL

Day-old Highline cockerels or mixed sex crossbred chicks (Nichols New Hampshire males $\times$ Crossbred females) obtained from a commercial hatchery were placed in electrically heated battery brooders and supplied feed and water ad libitum. The experimental period was 6 weeks. Twenty chicks

[1] Present address: Poultry Department, Washington State College, Pullman.

per group were started on each diet and the test was repeated at least once. In some cases it was repeated a second and third time.

Two basal diets, which were adequate in all the vitamins required by the chick, were used. Diet 1 was composed of a mixture of 40% of milo and soybean meal and 60% of a synthetic diet. This diet was similar to that used by Richardson, Hogan and Kempster ('45) to study the critical vitamins in rations for baby chicks. It was low, without the addition of crystalline

TABLE 1

Composition of basal diets

CONSTITUENTS	DIET NO.		AMOUNTS OF VITAMINS PER 100 GM [1]	
	1	2		
	%	%		mg
Ground milo	22.0	66.5	Alpha-tocopherol	2.5
Cerelose	36.2		Thiamine hydrochloride	0.4
Soybean meal	15.0	30.0	Riboflavin	0.4
Crude casein	14.5		Pyridoxine	0.4
Gelatin	3.0		Calcium pantothenate	2.0
Wood pulp	1.0		Niacin	5.0
Steamed bone meal	2.0	2.0	Choline chloride	200.0
NaCl	0.3	0.5	Folic acid	0.2
Mineral mixture [2]	2.0		Vitamin B_{12}	0.001
$MnSO_4 \cdot H_2O$	0.04	0.04	Menadione	2.5
Wesson oil	3.2	0.2		
Fish liver oil	0.8	0.8		

[1] The same vitamin mixture was added to both diets.

[2] Richardson and Hogan ('46).

vitamins, in pyridoxine, pantothenic acid, riboflavin, choline and niacin, and it was used to study the effects of suboptimum levels of these vitamins on the free amino acid content of the plasma. Diet 2 was a milo and soybean meal diet and it was used to study the influences of vitamin B_{12}. Crystalline vitamins were added to both diets to supply all the vitamins in amounts which were adequate for a rapid rate of growth in chicks. The composition of these diets is given in table 1.

When a suboptimum level of a vitamin was studied, it was either omitted from the diet or added at a low level. The total

amount supplied in the diet was sufficient to allow the chicks to grow large enough so that 10 ml of blood could be drawn by heart puncture from each chick.

In order to insure, as far as possible, that the chicks were actively assimilating amino acids at the time the blood was drawn, precautions were taken to see that they had access to feed at all times, and particularly immediately before the blood was drawn. Each group was divided into two subgroups at 6 weeks in such a manner that the average weight of the subgroup was the same as that of the original group. Blood was drawn by heart puncture, and 10 ml from each chick in the subgroup were combined in a 250-ml Erlenmeyer flask containing 20 mg of sodium heparinate per 100 ml of blood to prevent coagulation. This amount of sodium heparinate was dissolved in 1 ml of normal saline. Four samples of blood for each diet were obtained in this manner. Rinsing the syringe with a small amount of the heparin solution just before the sample was taken helped to prevent the blood from coagulating in the syringe. Eighty milliliters of the blood were centrifuged for 15 minutes at 1,800 r.p.m., and the plasma layer was drawn off by suction and stored at — 29°C. The protein-free filtrates containing the free amino acids were prepared, within a period of one to 6 weeks, by the procedure described by Heir and Bergeim ('45), and they were stored in stoppered 25 × 200 mm culture tubes at — 29°C. until the assays were completed. One milliliter of the protein-free filtrate was equivalent to 0.33 ml of the original plasma.

Arginine, lysine, methionine, tryptophan and valine were determined in the protein-free filtrates by microbiological assay. *Leuconostoc mesenteroides* was used as the test organism for arginine and lysine (Dunn et al., '44) and for methionine (Lyman et al., '46). *Streptococcus faecalis* was used for tryptophan (Kuiken et al., '47) and *Lactobacillus arabinosus* for valine (Kuiken et al., '43). The total volume in each tube of the microbiological assays was 2.5 ml. The use of this small volume for each amino acid made it possible to determine several amino acids on the protein-free filtrate which was

available. The assays were set up at two levels in duplicate, and the amount of an amino acid was calculated as the L form from a standard curve which was obtained with each series. The titrations were carried out directly in the assay tubes with 0.05 N sodium hydroxide by a micromethod similar to that described by Henderson et al. ('48). The device described by them was modified to the extent that a 5-ml microburette with a conventional stopcock was used instead of a solenoid apparatus, and a Beckman model G pH meter was used instead of an ordinary galvanometer.

Influence of the diet on the free amino acid content of the plasma

The average concentration of each of the 5 amino acids in the plasma of chicks which received diets 1 and 2 and the calculated amounts in the diets are given in table 2. These data were obtained when the diets contained adequate amounts of all the vitamins required by the chick.

It is seen that although the chicks grew at essentially the same rate on both diets, the level of the various amino acids in the plasma was quite different. Arginine was slightly lower in diet 2, but the amount of this amino acid in the plasma of chicks fed this ration was approximately twice that of chicks receiving diet 1. Likewise, tryptophan was lower in diet 2, but the plasma contained significantly more of this amino acid than that from chicks receiving diet 1. Lysine, methionine and valine were higher in diet 1 and the concentration of these amino acids were higher also in the plasma of the chicks which received this diet. However, the differences for valine were not significant.

The average amounts of the amino acids in the plasma and the number and average weights of the chicks from which blood was drawn when each of the vitamins was supplied at a suboptimum level are given in table 3. When the diet was low in pyridoxine or pantothenic acid, the concentration of each of the amino acids in the plasma, with the exception of lysine, was higher than when the diet was adequate in these vitamins.

TABLE 2

The free amino acids in the plasma and the calculated amounts in the diets

DIET NO.	NO. OF CHICKS	AVE. WT. OF CHICKS	PROTEIN IN DIET	ARGININE		LYSINE		METHIONINE		TRYPTOPHAN		VALINE	
				Diet	Plasma	Diet	Plasma	Diet	Plasma	Diet	Plasma	Diet	Plasma
		gm	%	%	*μg/ml*	%	*μg/ml*	%	*μg/ml*	%	*μg/ml*	%	*μg/ml*
1	84	515	23.7	1.261	49	1.423	110	0.566	48	0.291	62	1.409	191
2	39	515	20.0	1.229	94	0.975	69	0.321	34	0.265	76	1.038	176

TABLE 3

Effect of dietary vitamins on the free amino acids in the plasma of chicks

VITAMIN SUPPLIED [1] AT LOW LEVEL	NO. OF CHICKS	WT. OF CHICKS		FREE AMINO ACIDS IN THE PLASMA — μG/ML									
				Arginine		Lysine		Methionine		Tryptophan		Valine	
		Ave.	Diff.	Ave.	Diff.	Ave.	Diff.	Ave.	Diff.	Ave.	Diff.	Ave.	Diff.
		gm	*gm*										
				Diet 1 (semisynthetic)									
Basal	84	515		49		110		48		62		191	
Pyridoxine	59	379	— 136 [2]	66	17 [2]	100	— 10	62	14 [2]	74	12 [2]	252	61 [2]
Pantothenic acid	51	491	— 24	76	27 [2]	124	14 [2]	58	10 [2]	73	11 [2]	236	45 [2]
Riboflavin	38	466	— 49 [2]	41	— 1	117	7	60	12 [2]	79	17 [2]	267	76 [2]
Choline	37	476	— 39	40	— 9 [2]	88	— 22 [2]	54	6 [3]	65	3	192	1
Niacin	39	445	— 70 [2]	45	— 4	98	— 12	53	5 [3]	71	9 [2]	191	0
				Diet 2 (soybean meal)									
Basal	39	515		94		69		34		76		176	
Vitamin B_{12}	40	445	— 70 [2]	79	— 15 [3]	56	— 13 [2]	27	— 7 [3]	64	— 12 [2]	138	— 38 [2]

[1] Amounts of vitamins added to the basal diets are given in table 1. When pyridoxine, choline, niacin or vitamin B_{12} was studied at a low level, none of the vitamin was added. When pantothenic acid or riboflavin was studied, the amount added was 0.3 and 0.1 mg/100 gm, respectively.

[2] Significant at 1% level. Statistical analyses by use of the "t" test.

[3] Significant at 5% level. Statistical analyses by use of the "t" test.

When it was low in riboflavin, the concentration of methionine, tryptophan and valine was greater than when it contained an adequate amount of this vitamin. Arginine and lysine were not affected significantly by low riboflavin. With a diet low in choline, the plasma was lower in both arginine and lysine, and with a diet low in niacin, it was higher in tryptophan than with a diet which contained adequate amounts of these vitamins. With a diet low in vitamin B_{12}, the plasma contained a significantly smaller amount of each of the 5 amino acids than when it was adequate in this vitamin. Charkey et al. ('50) found a higher concentration of non-protein nitrogen and of certain free amino acids in the plasma of chicks which received a diet deficient in vitamin B_{12} than when they received a diet containing adequate amounts of this vitamin. The latter investigators used chicks which had been depleted of vitamin B_{12} and they used the same organism, *Streptococcus faecalis,* in the assay for each amino acid. The variations in the experimental procedures may account for the differences in the results.

The results of the studies reported in this paper show that the concentration of the free amino acids in the plasma of chicks may be influenced by the amount of a vitamin in the diet, but similar information is needed on other vitamins and amino acids before the data can be properly evaluated. It is possible that this approach may give some insight into the interrelationship of vitamins and amino acids in the formation of animal tissue.

SUMMARY

The influence of the amount of a vitamin in the diet on the free amino acid content of the blood plasma of chicks was investigated. The plasma of chicks receiving two basal diets, which were adequate in all the vitamins required by the chick, was assayed for free arginine, lysine, methionine, tryptophan and valine by microbiological procedures. Plasma from chicks receiving diets low in pyridoxine, pantothenic acid, riboflavin, niacin, choline and vitamin B_{12}, respectively, was assayed also for these amino acids. Chicks receiving the different basal

diets grew at about the same rate, but the concentration of amino acids in the plasma was different.

The soybean meal diet (diet 2) contained a smaller amount of the amino acids than the semisynthetic diet (diet 1) but plasma from the chicks which received the soybean meal diet contained twice as much arginine and significantly more tryptophan than that from those receiving the semisynthetic diet. When the diet was low in pyridoxine and pantothenic acid, the concentration of arginine, methionine and tryptophan in the plasma was increased. When it was low in riboflavin, the concentration of methionine, tryptophan and valine increased. When it was low in niacin, the concentration of arginine and lysine decreased. When it was low in vitamin B_{12}, the concentration of all 5 of the amino acids was decreased. These effects were analyzed statistically and found to be highly significant.

ACKNOWLEDGMENTS

Riboflavin, pyridoxine, calcium pantothenate, niacin, choline, crystalline vitamin B_{12}, alpha-tocopherol and menadione were generously furnished by Merck and Company through the courtesy of Dr. L. Michaud, and the folic acid was generously donated by Lederle Laboratories through the courtesy of Dr. T. H. Jukes.

LITERATURE CITED

CHARKEY, L. W., H. S. WILGUS, A. R. PATTON AND F. X. GASSNER 1950 Vitamin B_{12} in amino acid metabolism. Proc. Soc. Exp. Biol. Med., *73:* 21.

DUNN, M. S., S. SHANKMAN, M. N. CAMIEN, W. FRANKL AND L. B. ROCKLAND 1944 Investigations of amino acids, peptides and proteins. XVII. The amino acid requirement of *Leuconostoc mesenteroides* P-60. J. Biol. Chem., *156:* 703.

HEIR, S. W., AND O. BERGEIM 1945 Microbiological determinations of free leucine, isoleucine, valine and threonine in dog plasma. Ibid., *163:* 129.

HENDERSON, L. M., W. L. BRICKSON AND E. E. SNELL 1948 A micromethod for the microbiological determination of amino acids. Ibid., *172:* 31.

KUIKEN, K. A., C. M. LYMAN AND F. HALE 1947 Factors which influence the stability of tryptophan during the hydrolysis of proteins in alkaline solutions. Ibid., *171:* 551.

KUIKEN, K. A., W. H. NORMAN, C. M. LYMAN, F. HALE AND L. BLOTTER 1943 The microbiological determination of amino acids. I. Valine, leucine and isoleucine. Ibid., *151:* 615.

LYMAN, C. M., O. MOSELEY, S. WOOD AND F. HALE 1946 Note on the use of hydrogen peroxide-treated peptone in media for microbiological determination of amino acids. Arch. Biochem., *10:* 427.

RICHARDSON, L. R., AND A. G. HOGAN 1946 Diet of mother and hydrocephalus in infant rats. J. Nutrition, *32:* 459.

RICHARDSON, L. R., A. G. HOGAN AND H. L. KEMPSTER 1945 Critical vitamins in rations for baby chicks. Mo. Agr. Exp. Sta. Res. Bull. 390.

A QUANTITATIVE STUDY OF RUMEN SYNTHESIS IN THE BOVINE ON NATURAL AND PURIFIED RATIONS

I. PROTEIN, DRY MATTER AND NON-PROTEIN NITROGEN [1]

I. P. AGRAWALA,[2,3] C. W. DUNCAN AND C. F. HUFFMAN
Departments of Dairy and Agricultural Chemistry, Michigan State College, East Lansing

(Received for publication May 8, 1952)

Protein concentrates are expensive components of animal feeds. The substitution of some non-protein nitrogenous compound may supply nitrogen for ruminants more economically, provided microbial utilization is qualitatively as well as quantitatively efficient. Armsby ('11) concluded that non-protein nitrogenous compounds could serve as a partial substitute for protein in the ration of cattle. Bartlett and Cotton ('38) used heifers to study the average daily gains in body weight over a 142-day period. The heifers on a low-protein ration gained 0.99 lb. per day and those on a ration in which 30% of the total nitrogen of a normal ration was replaced with urea gained 1.23 lb. per day, whereas those on a normal protein ration gained 1.39 lb. per day. Hart et al. ('39) obtained similar results with calves when the ingested urea or ammonium bicarbonate did not exceed 40% of the dietary nitrogen. Briggs et al. ('46) replaced from 25 to 50% of the nitrogen in cottonseed meal with urea and obtained satisfactory growth in beef

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article 1356.

[2] This article is part of a dissertation presented to the Faculty of the Graduate School of Michigan State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Government of India Scholar. Present address: Indian Veterinary Research Institute, Izatnagar, U. P., India.

steers. Leitch and Davidson ('49) found from the ingestion of urea nitrogen[15] by beef calves that the proteins of liver, kidney and blood contained nitrogen[15] in excess of normal.

Satisfactory milk production was maintained when 25% of the nitrogen in an adequate ration was replaced with either blood meal or urea (Owen et al., '43). Rupel et al. ('43) maintained 15 milking cows on a linseed meal ration and an equal number of cows on a urea ration for their entire lactation periods and found no significant difference in the total amount of milk produced or in body weight. The nitrogen intakes due to linseed meal and urea comprised 45% of the total nitrogen ingested. Willett et al. ('46) reported that dairy cows were able to utilize urea nitrogen for milk production, but not as efficiently as nitrogen derived entirely from plant sources.

The literature supports the thesis that urea can function as a partial replacement for natural protein in the nutrition of ruminants, but no one has tested the efficiency of urea as the sole source of dietary nitrogen for calves. This investigation was undertaken to obtain quantitative information on the biosynthesis of protein in the rumen of fistulated calves when urea was the only source of dietary nitrogen. Information was also obtained on the amount and rate of disappearance of dry matter and non-protein nitrogen from the rumen.

EXPERIMENTAL

Three rumen-fistulated calves and one intact calf were used in the investigation. The calves were approximately 6 months of age at the start of the experiment and weighed from 400 to 560 lb. Each of the fistulated calves was fitted with a solid plastic plug which could be removed easily when samples were desired.

The compositions of the natural and purified rations are shown in table 1. The calves were fed once and watered twice daily. All of the calves received from 7 to 9 lb. of the natural ration for 15 days before they were changed to the purified ration. Theoretically, the quantity of feed fed was sufficient to meet the protein and calorific requirements of the calf.

Calf C711 was changed abruptly from the natural ration to 7 lb. of the purified ration per day. Rumen samples were taken on the 12th day. C714 was changed gradually from the natural to the purified ration over a 30-day period. Rumen samples were taken after the calf had been on the purified ration for 7 days. The above procedure was repeated 4 more times with C714. Calf C707 was changed gradually over a 45-day period from 9 lb. of the natural ration to 9 lb. of the purified ration per day. Rumen samples were taken 7 days after the calf had been on the purified ration. The intact calf was also changed gradually over a 45-day period from the normal to the purified ration.

TABLE 1

Composition of the rations

Natural ration — Protein equivalent (N × 6.25) = 10.8%

Brome-timothy hay	60%
Grain mixture	40%

(corn 36.5%, oats 36.5%, linseed oil meal 26.0%, NaCl 1.0%)

Purified ration — Protein equivalent (N × 6.25) = 12.0%

Cornstarch	42%	Cellophane	20%	Mineral mixture[1]	5%
Glucose	25%	Lard	4%	Urea	4%

Vitamins A and D: each calf received 400,000 I.U. of vitamin A and 5,000,000 I.U. of vitamin D every two weeks.

[1] Mineral mixture (%): $CaHPO_4 \cdot 2H_2O$, 11.0; $CaCO_3$, 16.0; K_2HPO_4, 27.0; $MgSO_4 \cdot 7H_2O$, 15.0; Na_2SO_4, 5.0; NaCl, 22.6; $FeC_6H_5O_7 \cdot 5H_2O$, 2.5; $MnSO_4 \cdot 4H_2O$, 0.7; KI, 0.08; $ZnCl_2$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.03; $CoSO_4 \cdot 7H_2O$, 0.07.

On the days when samples were taken, the entire rumen contents were removed through the fistula before the calf was fed (0-hr. sample). The evacuated contents were weighed, thoroughly mixed and a 500-gm aliquot was saved for analysis. The remaining contents were replaced immediately in the rumen and the calf was fed. Except for feeding, the same procedure was repeated 6 hours later (6-hr. sample). Bacterial and enzymatic action in the aliquot was inhibited by the addition of 500 ml of 95% ethyl alcohol. The samples were taken to the laboratory as soon as possible and evaporated to dryness in a

forced-hot-air oven at 60°C., ground to pass a 20-mesh sieve and stored in brown glass bottles until analyzed. The feed samples were dried, ground and stored in the same manner. The pH determinations were made immediately on fresh rumen liquid by the use of a Beckman pH meter.

Total nitrogen was determined by the Kjeldahl method. Non-protein nitrogen was determined by precipitating the protein in a 10-gm sample by mixing with 200 ml of 5% trichloroacetic acid in a Waring Blendor for 10 minutes. The mixture was centrifuged and the decanted portion was mixed with 10 gm of celite and filtered with the aid of suction. The nitrogen in a 50-ml portion was determined as above.

RESULTS AND DISCUSSION

The lack of hay in the purified ration of the fistulated calves resulted in the absence of rumination and the calves developed anorexia within 9 to 15 days, although the intact calf retained its appetite and appeared healthy after 21 days. The calves apparently could not survive on the purified ration, not only because the urea was converted into ammonia more rapidly than the rumen microorganisms could utilize it, but also because the high ammonia content may have been detrimental to the normal rumen population. Many ruminants are exacting in their requirements for amino acids (natural protein), and a ration which supplies little or no nitrogen in that form would lead to the disappearance of specific species of rumen microorganisms (McDonald, '52). Loosli et al. ('49) reported that intact lambs and goats fed a purified ration which contained urea as the only source of nitrogen remained healthy and gained in body weight over a period of three months. Mead and Goss ('35) maintained heifers on a roughage-free ration for 18 months and found that the digestibility of the ration was not significantly lower than the calculated value for the same mixture fed with roughage. The results obtained with the fistulated calves indicate also that cellophane is not a suitable substitute for natural roughage because cellulose deriva-

tives, in general, exhibit higher resistance to microbiological degradation than the cellulose molecule itself.

The data relative to the weights of the total rumen contents obtained for each trial before and 6 hours after feeding, and the percentages of dry matter, crude protein, non-protein nitrogen and pH, are compiled in table 2. The percentage of crude protein 6 hours after feeding the purified ration was higher in

TABLE 2

Weight and composition of the rumen contents of the calves on the purified and normal rations

TRIAL NO.	CALF NO.	RATION	WEIGHT OF RUMEN CONTENTS	DRY MATTER	CRUDE PROTEIN	NPN	pH
			gm	%	%	%	
			0-hr. samples				
1	C711	Purified	14,855	17.6	6.83	0.050	7.32
2	C714	Purified	15,224	10.2	3.34	0.022	8.06
3	C714	Purified	14,827	10.9	6.41	0.045	7.30
4	C714	Purified	16,613	10.0	4.48	0.045	6.40
5	C707	Purified	24,268	8.1	7.77	0.056	7.94
6	C714	Purified	12,757	11.2	4.72	0.028	8.12
7	C714	Purified	14,855	13.4	5.39	0.017	7.68
8	C714	Normal	13,437	15.6	16.32	0.135	6.88
			6-hr. samples				
1	C711	Purified	17,038	22.7	6.41	0.185	4.50
2	C714	Purified	22,793	12.9	5.97	0.168	4.93
3	C714	Purified	21,206	13.2	7.94	0.281	4.51
4	C714	Purified	22,113	12.7	8.12	0.281	4.32
5	C707	Purified	28,662	13.1	6.89	0.281	5.13
6	C714	Purified	18,640	15.4	4.54	0.129	6.32
7	C714	Purified	23,644	13.9	7.29	0.129	4.83
8	C714	Normal	22,084	15.9	17.38	0.154	5.28

only 4 of 7 trials than before feeding. Mills et al. ('42) found that the crude protein in the rumen at 6 hours after feeding a ration of hay plus starch was 6.9%, but the addition of urea to the ration increased the percentage to 10.7. The necessity of adding starch to rations containing urea has been demonstrated (Pearson and Smith, '43; Mills et al., '44). The purified ration used in this work contained ample starch, but the

percentage of crude protein in the rumen ingesta of the animals with a fistula was only one-third to one-half as much as that in the ingesta of the calf on the natural ration, although the nitrogen intake was greater than the protein nitrogen intake of the latter calf.

The percentage of non-protein nitrogen in the ingesta of the calves on the purified ration was markedly less than that in the ingesta of the calf on the natural ration at the 0-hr. collection, but the percentage was approximately equal to that in the ingesta of the calf on the natural ration at the 6-hr. collection. These findings are in agreement with those of Wegner et al. ('41), who found that the non-protein nitrogen in the rumen of a heifer fed a basal ration plus urea decreased within 4 to 6 hours to approximately the same level as that obtained from the basal ration.

The pH values of the rumen ingesta before feeding were comparable to those reported by Monroe and Perkins ('39) and Smith ('41), but the values obtained for the experimental calves at 6 hours were lower, indicating rapid fermentation and the production of acids. This observation is in agreement with that of El-Shazly ('52), who found that the evolution of ammonia in the rumen of sheep was in direct proportion to the amount of volatile fatty acids produced.

The rate of passage of dry matter from the rumen within a 6-hr. period was approximately the same on both the purified (approximately 60%) and natural (55%) rations. These values are substantially higher than those reported by Rathnow ('38), who found by the iron indicator method that 32.1, 48.5 and 58.8% of the dry matter passed from the rumen in three, 6 and 9 hours after feeding. Hale et al. ('47) found that 22.1% of the dry matter passed from the rumen in 6 hours when the lignin ratio method was used. When the same method of calculation that was used in this investigation was applied to the data obtained by Hale et al., a value of 55.7% was obtained, which is in good agreement with the value obtained for the calf on the natural ration.

Table 4 shows that approximately 90% of the non-protein nitrogen disappeared from the rumen within 6 hours after feeding. The amount of urea that was converted into ammonia and absorbed by the tissues or utilized in the biosynthetic activity of the microorganisms, or was excreted in the urine and feces, is not known. McDonald ('52) has shown that sheep can convert 20% of the nitrogen in a casein diet into ammonia within 4 hours after feeding. Owen et al. ('43) have shown that a large excess of urea + ammonia nitrogen appears in the urine and feces when milking cows ingest 25% of the total

TABLE 3

Dry matter (D.M.) removed from the rumen in 6 hours

TRIAL NO.	WEIGHT OF RUMEN D.M.	D.M. INTAKE	TOTAL	WEIGHT OF RUMEN D.M.	D.M. REMOVED
	gm	*gm*	*gm*	*gm*	% [1]
		0-hr. samples		*6-hr. samples*	
1	2,614	3,175	5,789	3,868	60.5
2	1,553	3,175	4,728	2,940	56.3
3	1,616	3,175	4,791	2,799	62.7
4	1,661	3,175	4,836	2,808	63.9
5	1,966	4,082	6,048	3,755	56.2
6	1,429	3,175	4,604	2,871	54.6
7	1,991	3,175	5,166	3,287	59.2
8	2,096	3,175	5,271	3,511	55.4

[1] Based on dry matter intake.

nitrogen intake as urea. The results obtained in this investigation indicate that only a small proportion of the urea nitrogen was utilized in the synthesis of protein, because more elementary nitrogen was present in the purified ration than was present in the natural ration.

The difficulties inherent in a critical interpretation of some percentage data are apparent from an examination of the crude protein data in table 2. In order to make a quantitative interpretation of these data, table 5 is presented to show the actual amounts of crude protein and "true" protein (total nitrogen — non-protein nitrogen × 6.25) that were present in the ingesta before and 6 hours after feeding. In all cases, there was

more "true" protein in the rumen 6 hours after feeding the purified ration than before. These amounts varied from 33 to 109 gm. Theoretically, these amounts only represent a part of the total amount synthesized, because some protein was removed from the rumen with the continual passage of dry matter; they cannot be compared with the apparent increase recorded for the calf on the natural ration. This calf obtained 345 gm of protein in the ration and, on the basis of the ingested protein, 27% had disappeared from the rumen within 6 hours after feeding. This again is a minimum value because it fails to

TABLE 4

Disappearance of non-protein nitrogen from the rumen dry matter in 6 hours

TRIAL NO.	NPN IN D.M.	UREA-N IN FEEDS	TOTAL	NPN IN D.M.	NPN REMOVED
	gm	*gm*	*gm*	*gm*	% [1]
		0-hr. samples		*6-hr. samples*	
1	1.3	59.2	60.5	7.2	90.0
2	0.3	59.2	59.5	4.9	92.2
3	0.7	59.2	59.9	7.9	87.8
4	0.8	59.2	60.0	7.9	88.0
5	1.1	76.1	77.2	10.6	87.5
6	0.4	59.2	59.6	3.7	94.4
7	0.3	59.2	59.5	4.2	93.4
8	2.8	0	2.8	5.4	..

[1] Based on urea-nitrogen intake.

take into consideration the amount of protein synthesized by the rumen microorganisms. On the basis of *in vitro* rumen synthesis, Pearson and Smith ('43) calculated that 450 gm of protein would be synthesized in a 75-kg rumen in 24 hours.

The data presented here are offered as evidence that an appreciable synthesis of protein occurred in the bovine rumen when urea was the only source of dietary intake. It is assumed that the rumen microorganisms were responsible for the increases obtained in protein content. This assumption is substantiated by the *in vitro* studies of Pearson and Smith ('43), who demonstrated the inhibitory action of sodium fluoride on rumen microorganisms, and by Black et al. ('52), who con-

cluded that the rumen microorganisms furnish the essential amino acids and that the tissues of the cow synthesize the nonessential amino acids.

There remain two significant groups of questions which should be pointed out in order to round out the problems involved in the study of rumen synthesis. The first concerns the interpretative limitations that are involved when dealing with a moving system; the other relates to the relevance of data obtained from the use of purified rations as compared to those depicting the behavior of rumen microorganisms under natural

TABLE 5

The amounts of "true" protein synthesized from urea in 6 hours

TRIAL NO.	TOTAL CRUDE PROTEIN	TOTAL NPN [1]	DIFFERENCE	TOTAL CRUDE PROTEIN	TOTAL NPN [1]	DIFFERENCE	INCREASE IN PROTEIN IN 6 HOURS
	gm	gm	gm	gm	gm	gm	gm
	0-hr. samples			*6-hr. samples*			
1	178.5	8.2	170.3	247.9	44.7	203.2	32.9
2	51.9	2.1	49.8	175.5	30.9	144.6	94.8
3	103.6	4.5	99.1	222.2	49.2	173.0	73.9
4	74.4	4.7	69.7	228.0	49.3	178.7	109.0
5	152.8	6.9	145.9	258.7	65.9	192.8	46.9
6	67.4	2.5	64.9	130.3	23.1	107.2	42.3
7	107.3	2.1	105.2	239.6	26.5	213.1	107.9
8	342.1	17.7	324.4	610.2	33.8	576.4	252.0

[1] Calculated as protein (N × 6.25).

conditions. Consequently, the conclusions drawn from this experiment are valid for only one specific set of conditions. It is highly probable that under these conditions the nitrogen nutrition of the microorganisms was the limiting factor, since 90% of the urea nitrogen was dissipated within 6 hours. If nitrogen compounds were available from which nitrogen could be liberated slowly, then the microorganism would have maximum opportunity to utilize the nitrogen. This approach shows great theoretical and practical possibilities, but it also indicates formidable difficulties which must be overcome, due to the complexity of dealing with a moving system.

SUMMARY

Seven balance trials were conducted with three rumen-fistulated calves maintained on a purified ration in which urea furnished the only source of dietary nitrogen and one trial was conducted with one calf maintained on a natural ration, to determine the rate of disappearance of dry matter and non-protein nitrogen from the rumen and to study the biosynthesis of protein.

The entire rumen contents were evacuated, weighed, sampled and replaced in the rumen both before and 6 hours after feeding. On a percentage basis, rumen dry matter and non-protein nitrogen increased appreciably within 6 hours after feeding, but crude protein increased in only 4 of 7 trials.

The rate of removal of dry matter was approximately the same irrespective of the ration fed (approximately 60%), and 90% of the non-protein nitrogen had disappeared from the rumen within 6 hours after feeding.

The data indicate an appreciable synthesis of protein from urea in the bovine rumen within 6 hours after feeding. In 7 trials, the amount of "true" protein synthesized varied from 33 to 109 gm. The increase in protein is assumed to be due to the biosynthetic activity of rumen microorganisms.

LITERATURE CITED

ARMSBY, H. P. 1911 The nutritive value of the nonprotein of feeding stuffs. U. S. Department of Agriculture, Bur. Animal Ind., Bull. 139.

BARTLETT, S., AND A. G. COTTON 1938 Urea as a protein substitute in the diet of young cattle. J. Dairy Res., *9:* 263.

BLACK, A. L., M. KLEIBER AND A. H. SMITH 1952 Carbonate and fatty acids as precursors of amino acids in casein. J. Biol. Chem., *197:* 365.

BRIGGS, H. M., W. D. GALLUP, A. E. DARLOW, D. F. STEPHENS AND C. KINNEY 1946 The performance of urea as an extender of protein. J. Animal Sci., *5:* 393.

EL-SHAZLY, K. 1952 Degradation of protein in the rumen of sheep. I. Some volatile fatty acids, including branch-chain isomers, found *in vivo*. Biochem. J., *51:* 640.

HALE, E. B., C. W. DUNCAN AND C. F. HUFFMAN 1947 Rumen digestion studies. I. A method of investigating chemical changes in the rumen. J. Nutrition, *34:* 733.

HART, E. B., G. BOHSTEDT, H. J. DEOBALD AND M. I. WEGNER 1939 The utilization of simple nitrogenous compounds such as urea and ammonium bicarbonate by growing calves. J. Dairy Sci., *22:* 785.

LEITCH, L. C., AND W. M. DAVIDSON 1949 The nutritive value of nitrogenous compounds for ruminants. III. Synthesis of urea containing N^{15}. Sci. Agr., *29:* 189.

LOOSLI, J. K., H. H. WILLIAMS, W. E. THOMAS, F. H. FERRIS AND L. A. MAYNARD 1949 Synthesis of amino acids in the rumen. Science, *110:* 144.

MCDONALD, I. W. 1952 The role of ammonia in ruminal digestion of protein. Biochem. J., *51:* 86.

MEAD, S. W., AND H. GOSS 1935 Ruminant digestion without roughage. J. Dairy Sci., *18:* 163.

MILLS, R. C., A. N. BOOTH, G. BOHSTEDT AND E. B. HART 1942 The utilization of urea by ruminants as influenced by the presence of starch in the ration. Ibid., *25:* 925.

MILLS, R. C., C. C. LARDINOIS, I. W. RUPEL AND E. B. HART 1944 Utilization of urea and growth of heifer calves with corn molasses or cane molasses as the only readily available carbohydrate in the ration. Ibid., *27:* 571.

MONROE, C. F., AND A. E. PERKINS 1939 A study of the pH values of the ingesta of the bovine rumen. Ibid., *22:* 983.

OWEN, E. C., J. A. B. SMITH AND N. C. WRIGHT 1943 Urea as a partial protein substitute in the feeding of dairy cattle. Biochem. J., *37:* 44.

PEARSON, R. M., AND J. A. B. SMITH 1943 The utilization of urea in the bovine rumen. 3. The synthesis and breakdown of protein in rumen ingesta. Ibid., *37:* 153.

RATHNOW, H. D. 1938 Über das Verhalten des Eisens Nahrung während der Pansenverdauung des Schafes. Inaug. Diss., Munich.

RUPEL, I. W., G. BOHSTEDT AND E. B. HART 1943 The comparative value of urea and linseed meal for milk production. J. Dairy Sci., *26:* 647.

SMITH, V. R. 1941 *In vivo* studies of hydrogen ion concentrations in the rumen of the dairy cow. Ibid., *24:* 659.

WEGNER, M. I., A. N. BOOTH, G. BOHSTEDT AND E. B. HART 1941 Preliminary observations on chemical changes of rumen ingesta with and without urea. Ibid., *24:* 51.

WILLETT, E. L., L. A. HENKE AND C. MARUYAMA 1946 The use of urea in rations for dairy cows under Hawaiian conditions. Ibid., *29:* 629.

A QUANTITATIVE STUDY OF RUMEN SYNTHESIS IN THE BOVINE ON NATURAL AND PURIFIED RATIONS

II. AMINO ACID CONTENT OF MIXED RUMEN PROTEINS [1]

C. W. DUNCAN, I. P. AGRAWALA,[2,3] C. F. HUFFMAN AND R. W. LUECKE
Departments of Dairy and Agricultural Chemistry, Michigan State College, East Lansing

(Received for publication May 8, 1952)

Considerable interest has been shown in recent years in the chemical composition of bovine rumen ingesta, and especially in the extensive synthetic capacity of rumen microorganisms. Limited data have become available on the percentage composition of rumen ingesta, derived from various rations, in respect to crude protein, crude fiber, ether extractives and nonprotein nitrogen (Wegner et al., '41a, b; Mills et al., '42, '44; Pearson and Smith, '43a, b; Harris et al., '43; Hale et al., '40, '47), but little is known concerning the amino acid content of the mixed rumen proteins.

Loosli et al. ('49) demonstrated that the rumen microorganisms in intact sheep and goats could synthesize considerable quantities of the essential amino acids from urea and inorganic sulfur. McDonald ('48) fed a lysine-free zein ration to rumen- and duodenal-fistulated sheep and found that the rumen microorganisms contained 7% lysine in their mixed proteins. On the basis of lysine determinations on the duodenal ingesta and

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article 1357.

[2] This article is part of a dissertation presented to the Faculty of the Graduate School of Michigan State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Government of India Scholar. Present address: Indian Veterinary Research Institute, Izatnagar, U. P., India.

the fractions of total protein leaving the rumen, it was estimated that 40% of the zein had been converted into microbial protein. Johanson et al. ('49) reported that the protein from the rumen bacteria of sheep contained more methionine when the sheep were on green pasture than when they were on dry pasture.

The purpose of this paper is to provide quantitative data on the essential amino acid content of the mixed rumen proteins of fistulated calves maintained on a purified ration in which urea supplied the only essential source of dietary nitrogen. For comparative purposes, similar data also were obtained from a calf maintained on a natural ration.

EXPERIMENTAL

The data concerning the experimental calves, the composition of the rations and the collection of samples have been reported previously (Agrawala et al., '53).

The protein hydrolysates for the determination of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine were prepared according to the method of Stokes et al. ('45). One-gram samples of the fat-free, dried rumen ingesta were diluted with 20 ml of concentrated HCl and autoclaved for 8 hours at 15 lb. pressure. The hydrolysates were cooled, neutralized to pH 6.6 to 6.8 with 18 *N* NaOH, made up to 100 ml, filtered, covered with a few drops of toluene and stored in the refrigerator for subsequent analysis. The enzymatic digestion procedure employed for tryptophan was essentially that proposed by Wooley and Sebrell ('45). One-gram samples of dried rumen ingesta were weighed into 100-ml volumetric flasks, 20 mg of pepsin and 40 mg of 0.1 *N* H_2SO_4 were added and the flasks incubated at 37°C. for 24 hours with constant shaking. The contents were then transferred to 100-ml beakers, 3.0 gm of K_2HPO_4 were added and the pH was adjusted to 8.4 with 3 *N* NaOH. The solutions were transferred again to 100-ml volumetric flasks, 20 mg of trypsin were added and allowed to incubate at 40°C. for 24 hours with constant shaking. The contents were cooled, adjusted to pH 7.0, diluted

to 100 ml, centrifuged, filtered, preserved with toluene and stored in the refrigerator. The assays were run after the proper dilutions had been made.

DL-Configurations of isoleucine, leucine, methionine, phenylalanine, threonine and valine were used in the preparation of standards for these amino acids, but L-configurations were used for the other 4. The amino acid determinations were carried out microbiologically by using *Streptococcus faecalis* (9790), *Leuconostoc mesenteroides* P-60 (8042) and *Lactobacillus plantarum* 17-5 (8014). The media used in the various determinations were essentially the same as those described by Sauberlich and Baumann ('46), with the exception of those used for isoleucine and methionine, which were prepared according to the method of Kuiken et al. ('43) as modified by Lyman et al. ('46).

RESULTS AND DISCUSSION

The results obtained from the microbiological analysis of the amino acids in the "true" protein in the rumen dry matter are compiled in table 1. The most striking differences are shown in the results obtained for the calves on the purified ration as compared to those on the natural ration. The amino acid values obtained when the natural ration was fed were uniform at both collection periods and markedly higher than those obtained when the purified ration was fed. The tendency for the 6-hour samples in each trial with the experimental calves to be higher or lower than the corresponding zero-hour samples was apparently associated with the passage of ingesta from the rumen, but the trend appeared to be consistent for each trial. These data fail also to indicate definitely whether protein synthesis had occurred in the rumen during the first 6 hours after feeding, because the values were the same or lower in 4 of 7 trials. This observation is true for both rations and erroneous conclusions could be drawn unless the entire rumen contents had been removed and weighed.

The data recorded in table 2 show the actual number of grams of the individual amino acids present in the rumen at

zero time and 6 hours later. Since the purified ration was practically amino acid-free, the increase in the amino acid content is presumed to be the result of the biosynthesis of amino acids from urea nitrogen by the rumen microorganisms.

TABLE 1

Amino acid composition of the rumen dry matter before and 6 hours after feeding[1]

TRIAL NO.		1	2	3	4	5	6	7	8
Arginine	0	0.33	0.16	0.29	0.25	0.35	0.22	0.27	0.88
	6	0.25	0.25	0.31	0.31	0.25	0.19	0.32	1.00
Histidine	0	0.14	0.07	0.13	0.10	0.15	0.09	0.09	0.38
	6	0.12	0.10	0.13	0.10	0.10	0.08	0.12	0.39
Isoleucine	0	0.22	0.19	0.34	0.23	0.44	0.19	0.23	0.80
	6	0.22	0.29	0.34	0.28	0.32	0.12	0.31	0.86
Leucine	0	0.41	0.24	0.41	0.32	0.50	0.25	0.32	1.24
	6	0.36	0.33	0.41	0.38	0.35	0.21	0.38	1.28
Lysine	0	0.37	0.17	0.34	0.22	0.31	0.18	0.20	0.69
	6	0.34	0.26	0.41	0.33	0.21	0.17	0.36	0.63
Methionine	0	0.09	0.03	0.09	0.04	0.09	0.04	0.06	0.16
	6	0.08	0.06	0.08	0.07	0.07	0.03	0.09	0.17
Phenylalanine	0	0.22	0.11	0.23	0.16	0.26	0.15	0.17	0.98
	6	0.20	0.17	0.22	0.22	0.20	0.13	0.21	0.98
Threonine	0	0.27	0.20	0.33	0.22	0.39	0.22	0.28	0.73
	6	0.27	0.25	0.29	0.27	0.26	0.18	0.33	0.70
Tryptophan	0	0.04	0.02	0.04	0.03	0.04	0.02	0.02	0.10
	6	0.03	0.03	0.04	0.04	0.03	0.02	0.03	0.10
Valine	0	0.38	0.17	0.36	0.22	0.43	0.22	0.27	0.90
	6	0.30	0.27	0.37	0.31	0.31	0.19	0.34	0.89

[1] All values are expressed as a percentage of the sample on the fat- and moisture-free basis.

The average percentage increase in the amounts of amino acids synthesized between the zero- and 6-hour collections was approximately the same for both rations, except for trial 2, but the total number of grams of each amino acid in the rumen of the calf on the normal ration was markedly higher than that in the rumen of the calves on the purified ration. The number

of grams of amino acids ingested in the natural ration plus those already present in the rumen before feeding more than accounted for the increases obtained at the 6-hour collection.

In order to determine whether the mixed proteins in the rumen of the calves on the purified ration were similar in amino acid content to the mixed proteins in the rumen of the

TABLE 2

The grams of amino acids present in the rumen dry matter before and 6 hours after feeding

TRIAL NO.	TIME	ARG [1]	HIS	ISOL	LEU	LYS	MET	PHE	THR	TRY	VAL
	hr.										
1	0	8.6	3.7	5.8	10.7	9.7	2.4	5.8	7.1	1.1	9.9
	6	9.7	4.6	8.5	13.9	13.2	3.1	7.7	10.4	1.2	11.6
2	0	2.5	1.1	3.0	3.7	2.6	0.5	1.7	3.1	0.3	2.6
	6	7.4	2.9	8.5	9.7	7.6	1.8	5.0	7.4	0.9	7.9
3	0	4.7	2.1	5.5	6.6	5.5	1.5	3.7	5.3	0.7	5.8
	6	8.7	3.6	9.5	11.5	11.5	2.2	6.2	8.1	1.1	10.4
4	0	4.2	1.7	3.8	5.3	3.7	0.7	2.7	3.7	0.5	3.7
	6	8.7	2.8	7.9	10.7	9.3	2.0	6.2	7.6	1.1	8.7
5	0	6.9	3.0	8.7	9.8	6.1	1.8	5.1	7.7	0.8	8.5
	6	9.4	3.8	12.0	13.1	7.9	2.6	7.5	9.8	1.1	11.6
6	0	3.1	1.3	2.7	3.6	2.6	0.6	2.1	3.1	0.3	3.1
	6	5.5	2.3	3.5	6.0	4.9	0.9	3.7	5.2	0.6	5.5
7	0	5.4	1.8	4.6	6.4	4.0	1.2	3.4	5.6	0.4	5.4
	6	10.5	3.9	10.2	12.5	11.8	3.0	6.9	10.9	1.0	11.2
8	0	18.4	8.0	16.8	26.0	14.5	3.4	20.5	15.3	2.1	18.9
	6	35.1	13.7	30.2	44.9	22.1	6.0	34.4	24.6	3.5	31.3

[1] The first three letters of the amino acid are used as the symbol.

calf on the natural ration, the data were converted into grams of amino acids per 100 gm of "true" protein, and are recorded in table 3. It is recognized that data expressed in this way may be open to criticism, since they are obtained by calculation (Jones, '41), but they are useful for comparative purposes because all values are reduced to an equivalent basis. The calculated values obtained from the calves on the purified ration

show good agreement among the zero- and 6-hour samples, although a few varied by more than 15%. With the exception of those for phenylalanine, these values also show good agreement with those obtained from the calf on the natural ration, although there appears to be a constant small difference in the

TABLE 3

The amino acid content of the "true" protein in the dried rumen ingesta before and 6 hours after feeding[1]

TRIAL NO.	TIME	ARG[2]	HIS	ISOL	LEU	LYS	MET	PHE	THR	TRY	VAL
	hr.										
1	0	5.0	2.2	3.4	6.3	5.7	1.4	3.4	4.2	0.6	5.8
	6	4.8	2.3	4.2	6.8	6.5	1.5	3.8	5.1	0.6	5.7
2	0	5.0	2.2	6.0	7.4	5.2	1.0	3.4	6.2	0.6	5.2
	6	5.1	2.0	5.9	6.7	5.3	1.2	3.5	5.1	0.6	5.5
3	0	4.7	2.1	5.5	6.7	5.5	1.5	3.7	5.3	0.7	5.9
	6	5.0	2.1	5.5	6.6	6.6	1.3	3.6	4.7	0.6	6.0
4	0	6.0	2.4	5.5	7.6	5.3	1.0	3.9	5.3	0.7	5.3
	6	4.9	1.6	4.4	6.0	5.2	1.1	3.5	4.3	0.6	4.9
5	0	4.7	2.1	6.0	6.7	4.2	1.3	3.5	5.3	0.5	5.8
	6	4.9	2.0	6.2	6.8	4.1	1.3	3.9	5.1	0.6	6.0
6	0	4.8	2.0	4.2	5.5	4.0	0.9	3.2	4.8	0.5	4.8
	6	5.1	2.1	3.3	5.6	4.6	0.8	3.4	4.9	0.6	5.1
7	0	5.1	1.7	4.4	6.1	3.8	1.1	3.2	5.3	0.4	5.1
	6	4.9	1.8	4.8	5.9	5.5	1.4	3.2	5.1	0.5	5.3
8	0	5.7	2.5	5.2	8.0	4.5	1.0	6.3	4.7	0.6	5.8
	6	6.1	2.4	5.2	7.8	3.8	1.0	6.0	4.3	0.6	5.4

[1] All values are expressed as grams per 100 gm of "true" protein on the nonprotein nitrogen-, fat- and moisture-free basis.

[2] The first three letters of the amino acid are used as the symbol.

arginine, leucine, lysine, and methionine content. Except for these particular amino acids, the amino acid contents of the mixed proteins derived from the two different rations appear to be fundamentally similar, but the question as to whether the apparent disagreements will be larger or smaller must wait until our knowledge of rumen proteins is enlarged.

Since the establishment of the empirical formula of proteins in terms of constituent amino acids is fundamental to an understanding of their composition, it was thought that calculation of the minimum molecular weight of the mixed proteins in the rumen of the calves on the two unlike rations might show some similarities, provided somewhat similar amounts of the same kind of protein were present. The data presented in table 4 show these calculations, and indicate the exceptional agreement among all of the 6-hour samples on both the purified

TABLE 4

The average minimum molecular weights of the "true" proteins in the rumen before and 6 hours after feeding

TRIAL NO.	CALF NO.	0-HOUR SAMPLES	6-HOUR SAMPLES
1	C711	68,410	68,263
2	C714	68,805	69,026
3	C714	58,470	68,382
4	C714	58,309	67,712
5	C707	80,961	68,082
6	C714	82,077	68,201
7	C714	102,696	81,872
8	C714	68,836	68,835

and natural rations and the fair agreement among the zero-hour samples. Since the calculations were based on the amino acid present in the smallest concentration, the apparent differences in the tryptophan content of the mixed proteins (table 1) were enough to account for the differences recorded in table 4. The method employed for making the calculations has been reported previously (Weinstein et al., '51).

Admittedly, minimum molecular weight calculations are more applicable when dealing with a pure protein, but the uniformity of the values obtained in this investigation suggests that the mixed proteins synthesized from urea are closely related to, or are components of, those synthesized by the naturally-occurring bovine rumen microorganisms.

SUMMARY

Mixed rumen proteins obtained from 7 trials with rumen-fistulated calves maintained on a purified ration in which urea supplied the only essential source of dietary nitrogen, and from one calf fed a natural ration, were assayed by microbiological methods for the amino acids known to be essential for the rat.

Quantitative evidence was obtained to show that rumen microorganisms can utilize urea nitrogen to synthesize amino acids. With the exception of histidine, the amino acid pattern of the mixed proteins in the ingesta of the calves on the purified ration was fundamentally similar to that found for the calf on the natural ration.

Minimum molecular weights calculated for the mixed rumen proteins of the calves on the purified ration were in agreement with the values obtained for the mixed proteins in the ingesta of the calf on the natural ration.

LITERATURE CITED

AGRAWALA, I. P., C. W. DUNCAN AND C. F. HUFFMAN 1953 A quantitative study of rumen synthesis in the bovine on natural and purified rations. I. Protein, dry matter and non-protein nitrogen. J. Nutrition, *49:* 29–40.

HALE, E. B., C. W. DUNCAN AND C. F. HUFFMAN 1940 Rumen digestion in the bovine with some observations on the digestibility of alfalfa hay. J. Dairy Sci., *23:* 953.

———— 1947 Rumen digestion studies. II. Studies in the chemistry of rumen digestion. J. Nutrition, *34:* 747.

HARRIS, L. E., S. H. WORK AND L. A. HENKE 1943 The utilization of urea and soybean oil meal nitrogen by steers. J. Animal Sci., *2:* 328.

JOHANSON, H., R. J. MOIR AND E. J. UNDERWOOD 1949 Sulfur-containing amino acids in the rumen bacteria of sheep. Nature, *163:* 101.

JONES, D. B. 1941 Factors for converting percentages of nitrogen in foods and feeds into percentages of protein. U. S. Department of Agriculture Circ. 183. Rev.

KUIKEN, K. A., W. H. NORMAN, C. M. LYMAN, F. HALE AND L. BLOTTER 1943 The microbiological determination of amino acids. I. Valine, leucine and isoleucine. J. Biol. Chem., *151:* 615.

LOOSLI, J. K., H. H. WILLIAMS, W. E. THOMAS, F. H. FERRIS AND L. A. MAYNARD 1949 Synthesis of amino acids in the rumen. Science, *110:* 144.

LYMAN, C. M., O. MOSELEY, B. BUTLER, S. WOOD AND F. HALE 1946 The microbiological determination of amino acids. III. Methionine. J. Biol. Chem., *166:* 161.

McDONALD, I. W. 1948 The extent of conversion of food protein to microbial protein in the rumen of sheep. J. Physiol., *107:* 21P.

MILLS, R. C., A. N. BOOTH, G. BOHSTEDT AND E. B. HART 1942 The utilization of urea by ruminants as influenced by the presence of starch in the ration. J. Dairy Sci., *25:* 925.

MILLS, R. C., C. C. LARDINOIS, I. W. RUPEL AND E. B. HART 1944 Utilization of urea and growth of heifer calves with corn molasses or cane molasses as the only readily available carbohydrate in the ration. Ibid., *27:* 571.

PEARSON, R. M., AND J. A. B. SMITH 1943a The utilization of urea in the bovine rumen. I. Methods of analysis of the rumen ingesta and preliminary experiments *in vivo*. Biochem. J., *37:* 142.

———— 1943b The utilization of urea in the bovine rumen. III. The synthesis and breakdown of protein in rumen ingesta. Ibid., *37:* 153.

SAUBERLICH, H. E., AND C. A. BAUMANN 1946 The effect of dietary protein upon amino acid excretion by rats and mice. J. Biol. Chem., *166:* 417.

STOKES, J. L., M. GUNNESS, I. M. DWYER AND M. C. CASWELL 1945 Microbiological methods for the determination of amino acids. II. A uniform assay for the ten essential amino acids. Ibid., *160:* 35.

WEGNER, M. I., A. N. BOOTH, G. BOHSTEDT AND E. B. HART 1941a Preliminary observations on chemical changes of rumen ingesta with and without urea. J. Dairy Sci., *24:* 51.

———— 1941b The utilization of urea by ruminants as influenced by the level of protein in the ration. Ibid., *24:* 835.

WEINSTEIN, B. R., C. W. DUNCAN AND G. M. TROUT 1951 The solar-activated flavor of homogenized milk. IV. Isolation and characterization of a whey constituent capable of producing the solar-activated flavor. Ibid., *34:* 570.

WOOLEY, J. G., AND W. H. SEBRELL 1945 Two microbiological methods for the determination of *l*(-)-tryptophane in proteins and other complex substances. J. Biol. Chem., *157:* 141.

EFFECTS OF PROLONGED PERCUTANEOUS ADMINISTRATION OF METHYL TESTOSTERONE AND ESTRADIOL ON GROWING MALE RATS [1]

AMOS E. LIGHT AND JOHN A. TORNABEN
The Wellcome Research Laboratories, Tuckahoe, N. Y.

TEN FIGURES

(Received for publication June 12, 1952)

INTRODUCTION

During recent years it has been shown that an upset of the balance between the male and female sex hormones in the mammalian body has pronounced effects on its development. The results of many investigators are summarized by Kochakian ('46), Emmens and Parkes ('47), Carter, Cohen and Shorr ('47) and Heller and Maddock ('47). Because of the increasing importance attached to these hormones in studying the process of aging (Goldzieher, '49; Benjamin, '51), it was believed that a more comprehensive metabolic study should be made, especially with male animals receiving continuous excessive dosages of these hormones over an extended period of time. The cutaneous route of administration was chosen because of possibly a more constant release of the hormones into the system (Zondek, '35; Moore, La Mar and Beck, '38) and because of interest in maximum dermal effects.

[1] Presented in part at the 16th annual meeting of the American Institute of Nutrition at New York, N. Y., April, 1951. See Proceedings of the Federation of American Societies for Experimental Biology, *11:* 1, part I, p. 448, 1952.

EXPERIMENTAL PROCEDURES

Three groups of recently weaned male rats (Carworth Farms), 5 animals per group, were placed in oblong cages and given an adequate diet of ground fox chow [2] and water ad libitum. The animal weights (36 to 42 gm at the start of the experiment) were recorded at weekly intervals and the food consumption three times per week. Water intake and urine and fecal output were likewise measured for intermittent periods. The protein and chloride contents of the urine samples were also determined (Light et al., '52).

Each animal in group E received approximately 200 mg of an ointment [3] containing 30 μg of estradiol, and each in group A received 200 mg of another ointment [4] containing 400 μg of methyl testosterone, while those in group C were treated with a placebo.[5] All ointments were applied daily by inunction into areas on the backs of the animals which were clipped at weekly intervals with an electric clipper (size 0000 head). At the start of the experiment these areas contained approximately 20 cm^2 of skin, but as the rats grew they became enlarged to some 75 to 125 cm^2, depending on the size of the animals. The hair obtained each week was weighed in order to determine the effects of the inuncted hormones on hair growth. Skin biopsies were obtained at different periods and the animals were autopsied after 201 days of continuous treatment. Weights and measurements of various tissues were obtained in addition to hematological and histological studies. The specific gravity of the empty carcass was determined by water displacement.

Although the ointments were administered to the animals by rubbing into the skin until practically no residue was visible, it must be admitted that an appreciable amount of active material may have been licked off and consumed orally by

[2] Purina.

[3] Progynon — DH. Schering Corp., Bloomfield, N. J.

[4] Oreton — M. Schering Corp., Bloomfield, N. J.

[5] Oil in water emulsion.

the rats. Since maximum effects due to continuous administration were desired, this did not interfere with the results obtained.

RESULTS

Growth

As illustrated in figure 1, it was found that estradiol markedly inhibited the growth of young male rats, whereas the methyl testosterone had no significant effect ("t" test, Snedecor, '46). The action of estradiol is in agreement with the results of Hooker and Pfeiffer ('43), who injected this hormone in rats over extended periods of time.

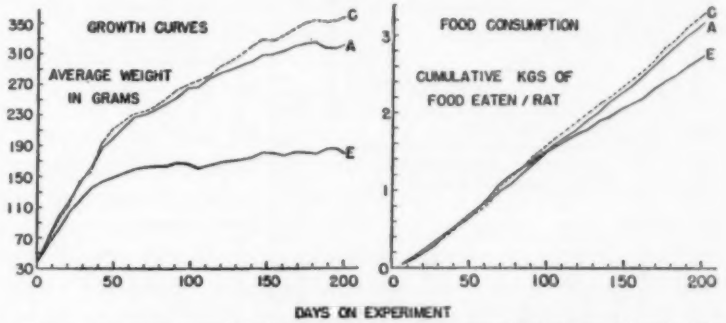


Fig. 1 Average growth and cumulative food consumption curves of male rats inuncted with estradiol (E), methyl testosterone (A) and placebo (C) ointments.

Food consumption

Although the total average food consumption (fig. 1) was less for the estradiol-treated animals, the amount eaten per kilogram of weight per day (fig. 2) was actually higher. This amplifies the introductory work by Korenchevsky and Dennison ('34). When the efficiency of food utilization for adding weight is considered, the female hormone-treated rats were only one-half as efficient as the others: 21.3 gm of food were required for the estradiol-treated animals to produce 1 gm gain in weight, whereas only 11.3 gm and 11.1 gm were needed by the methyl testosterone- and placebo-treated animals, re-

spectively. When specific weekly periods were observed (fig. 3), it was again noted that the food consumption per body weight was consistently higher for the estrogen-treated animals, but their gain in weight was exceedingly variable. Fecal excretion was more constant.

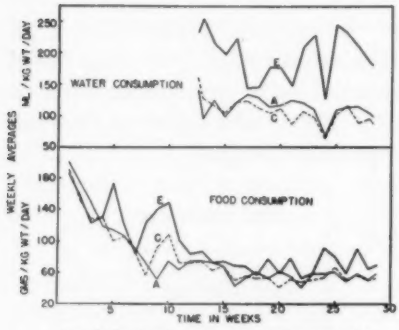


Fig. 2 Food and water consumption expressed in gm and ml per kg body weight per day – weekly averages plotted against time.

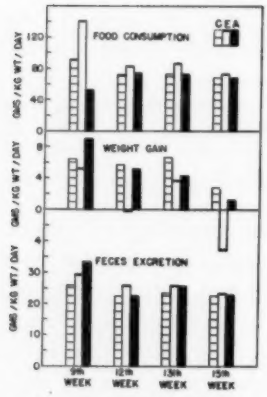


Fig. 3 Comparison of average food consumption, weight gain and fecal excretion during 4 weekly periods of the growth curve.

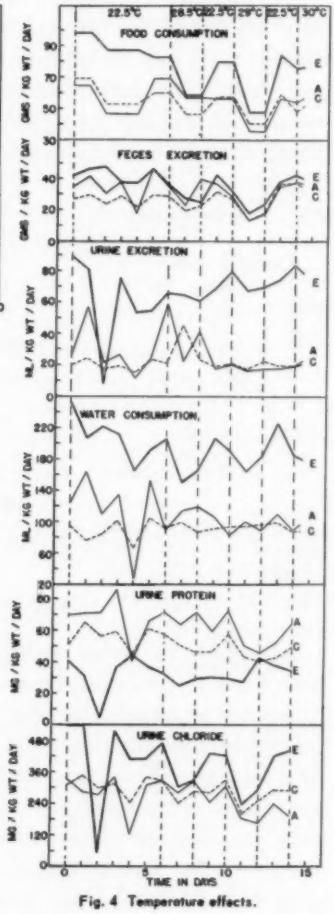


Fig. 4 Temperature effects.

Figures 2–4

Water consumption

The water consumption for the three groups of animals was recorded for the last 117 consecutive days of the experiment. When weekly averages in milliliters of water per kilogram of body weight per day were plotted against time (fig. 2), it became obvious that the estrogen-treated animals had extraordinary intakes of fluid.

Hair growth

Both hormones appeared to depress the growth of hair on the clipped areas, with estradiol possibly causing the most pronounced effects toward the end of the experiment (Ingle and Baker, '51). Dermatological changes will be described elsewhere.

Temperature effects

During the last 15 days of the experiment various controlled environmental temperature changes were made to determine the effects on these animals. Following a 6-day stabilizing period at 22.5°C., the temperature was raised to 28.5°C. for two days, then reduced again to 22.5°C. for two days. This was repeated during the next 4 days. It was found that the food consumption (fig. 4), especially of the estrogen-treated animals, was markedly reduced at the higher temperatures. Fecal excretion was parallel to food intake. The water intake of the estrogen-inuncted animals likewise decreased at the higher temperatures. The urine values were also larger but more constant and followed the water consumption with about a day's lag. It was interesting to note that the specific gravity of the urine from the estrogen-treated animals averaged 1.028, while that from the other two groups averaged 1.053. As has been noted elsewhere (Sellers, Goodman, Marmorston and Smith, '50; Linkswiler, Reynolds and Baumann, '52), the urine protein of estrogen-treated rats was lowered, whereas that of the androgen-treated animals was slightly increased. The amount of chloride excreted appeared to follow the food consumption curve.

Autopsy weights and measurements

From table 1 it may be seen that the body weights of the animals were depressed by estrogen inunction, while on the other hand the ratio of skeletal development to body weight was magnified. The pituitaries and adrenals were especially

TABLE 1

Summary of autopsy weights and measurements

	ESTRADIOL	METHYL TESTOSTERONE	CONTROLS
Weight, gm per rat	178.3[1] ± 18.3[2]	320.6 ± 58.7[2]	356.3 ± 25.0[2]
Body length (minus tail), cm per gm per rat	0.103[1] ± 0.007	0.067 ± 0.011	0.061 ± 0.004
Tail length, cm per gm per rat	0.093[1] ± 0.008	0.062 ± 0.009	0.055 ± 0.002
Specific gravity of carcass	1.080 ± 0.009	1.088[3] ± 0.008	1.071 ± 0.014
Pituitary, % body weight	0.0091[1] ± 0.0018	0.0035 ± 0.0006	0.0032 ± 0.0005
Adrenal, % body weight	0.0123[1] ± 0.0012	0.0051 ± 0.0013	0.0053 ± 0.0008
Testes, % body weight	0.111[1] ± 0.015	0.296 ± 0.040	0.365 ± 0.093
Kidney, % body weight	0.425[1] ± 0.022	0.521[1] ± 0.036	0.400 ± 0.013
Gastrocnemius muscle, % body weight	0.211[1] ± 0.014	0.187 ± 0.012	0.178 ± 0.010
Femur bone, % body weight	0.379[1] ± 0.028	0.318 ± 0.031	0.297 ± 0.007
G.I. tract and contents, % body weight	12.11[1] ± 0.90	9.17[1] ± 0.62	7.89 ± 0.96
Cervical lymph node, % body weight	0.105 ± 0.017	0.100 ± 0.015	0.088 ± 0.010
Thyroid and tracheal ring, % body weight	0.026[1] ± 0.001	0.018 ± 0.006	0.017 ± 0.004
Heart, lungs and thymus, % body weight	1.683[1] ± 0.463	1.179 ± 0.214	1.064 ± 0.091
Spleen, % body weight	0.205[1] ± 0.017	0.173 ± 0.012	0.177 ± 0.008
Liver, % body weight	4.22 ± 0.37	4.20 ± 0.43	4.65 ± 1.48

[1] Significantly different from controls at P = 0.05 level, using Fischer's formula for the "t" test (Snedecor, '46).

[2] Standard deviation.

[3] Significantly different at P = 0.055 level although not at P = 0.05 level.

enlarged in the estrogen animals, whereas the testicular development was markedly depressed. The anabolic effect (Kochakian, Robertson and Bartlett, '50) of the methyl testosterone was especially noticeable when the kidney weights and the specific gravity of the carcasses were studied. The increased percentage weights of the gastrocnemius muscle and the femur bone in the estrogen-treated males were likewise indicative of minimum fat deposition in the tissues. The significantly increased percentage weights of the gastrointestinal tract, including contents, emphasized greater food consumption, especially in the case of the estrogen-treated animals.

Wound healing

It was also interesting to observe that the healing rates of the biopsy cuts were increased by the androgens but delayed by the estrogens. The scabs on the healed cuts of the methyl testosterone-treated animals disappeared at the end of 5 to 6 days, and on the controls after about 7 days, while those on the estradiol-treated animals remained for as long as two weeks.

Hematology

The results of the hematological study (table 2) indicate that in the androgen-treated group the hemoglobin concentration was higher and the erythrocyte counts were lower than in the control group. However, in the estrogen-treated animals both hemoglobin and erythrocyte values were lower. No definite changes were observed in the different counts.

Histopathology

Preliminary histopathological studies were made of the spleens, adrenal cortices, testes, pituitaries, thyroids, kidneys and livers. A diffuse lymphatic tissue with modified germinal centers is seen in the spleens of the androgen-treated group (plate 1). There is also an increase in red pulp. The splenic follicles in the estrogen group are well defined. The adrenal cortices of the androgen animals are

normal except for a very slight lipoid depletion in the zona fasciculata. In the estrogen group the capsules are thickened, the zona glomerulosa is ill-defined, the cells are atrophic and there is a preponderance of pyknotic nuclei. There is a pronounced deposition of lipids in the zona fasciculata, as indicated by increased vacuolization. The sinusoids are dilated. All stages of spermatogenesis are present in the testicular tissues of the androgen-treated animals. However, in the

TABLE 2

Summary of hematology study

GROUP	GROUP AVERAGES		
	Hemoglobin	Erythrocytes	Leukocytes
	gm/100 ml blood	*millions/mm³*	*per mm³*
Control	13.80 ± 0.15 [1]	10.29 ± 0.15 [1]	9,825 ± 2,738 [1]
Methyl testosterone	14.90 [2] ± 0.16	9.15 [2] ± 0.7	9,320 ± 554
Estradiol	12.20 [2] ± 0.14	7.15 [2] ± 1.5	7,825 ± 1,565

Differential counts

GROUP	GROUP AVERAGES IN PER CENT VALUES						
	Polymorph.	Juven.	Total Neutro.	Lympho.	Mono.	Baso.	Eosino.
Control	25.75	0.00	25.75	71.50	0.25	0.00	2.50
Methyl testosterone	35.40	0.40	35.80	56.60	0.60	0.00	5.00
Estradiol	36.00	0.50	36.50	62.50	0.25	0.00	0.75

[1] Standard deviation.

[2] Significantly different from controls at P = 0.05 level on application of "t" test.

testes of the estrogen-treated animals the seminiferous tubules are grossly atrophic and a complete arrest of spermatogenesis is seen at the level of the spermatogonia. The few spermatogonia present are abnormal. This tissue is nonfunctional. There is an increase in basophils in the pituitaries of the androgen animals. The sinusoids in the anterior lobe are less dilated than in the controls. In the estrogen group the sinusoids are more dilated than in the controls, and there is a decrease in the basophilic elements. A few mitotic fig-

ures are seen in the epithelial portion of the glands. No changes are observed in the kidneys and livers of either group as compared to the controls. In general, it may be stated that the tissues taken from the androgen-treated animals presented a more normal histological picture, whereas estrogen application caused definite changes in many parts of the body.

DISCUSSION

From the results presented it appears that estrogen treatment of growing male rats induces a period of extreme energy expenditure which causes an increase in appetite for food and water but permits only subnormal growth. Since wound healing is delayed, this may suggest that the hormonal action is mediated through the pituitary and adrenals. Additional evidence for this is also obtained from the increased weights of these two glands. Of all the tissues studied at autopsy, these were the only two that increased in percentage of body weight as much as two and one-half to three times the values for the controls. In addition, the testes failed to descend and were markedly impaired in development. The growth of hair was likewise inhibited. These reactions may all be related to the stress syndrome of Selye ('50).

Changes in the food consumption of normal male rats due to different temperatures, including the range investigated here, have been reported by Brobeck ('48), but it is extremely interesting to observe the magnification of these changes induced by estradiol in the animals used in this study. When the temperature was increased, the quantity of food eaten was reduced much more in the case of the estrogen-treated animals. Likewise a larger intake was obtained with lowered temperatures. These marked variabilities in food and water intake and in energy expenditure following estrogen treatment are undoubtedly caused by the induced imbalances of the sex hormone relationship in these growing male rats. It would be extremely interesting to analyze human male development and behavior with these observations kept in mind. It appears that much research work is yet to be done

in the study of nutrition during periods of sex hormone imbalances (Meites, '52).

CONCLUSIONS

1. Growing male rats maintained on an adequate diet but inuncted with estradiol ointment were severely retarded in growth and development. However, when results were considered on a body weight basis the food and water intake were markedly increased, indicating a greater expenditure of energy with low efficiency in its utilization in adding muscle and fat tissue. However, the skeletal development showed a percentagewise increase. It may be postulated that these effects were mediated through the pituitaries and adrenals. The healing of wounds was likewise retarded and the urinary protein was reduced.

2. Decided variabilities in food and water consumption were also obtained in estrogen-treated animals following temperature changes. The intakes increased during periods of lower temperatures and decreased with warmer environments.

3. On the other hand, the inunction of methyl testosterone was associated with enlargement of the kidneys and possibly increased deposition of protein tissue. This treatment likewise shortened the time required for wound healing.

ACKNOWLEDGMENTS

The authors wish to take this opportunity to express their thanks for the cooperation of Dr. E. J. de Beer, Acting Director of The Wellcome Research Laboratories, and for the encouragement given by Dr. G. R. Cowgill, Yale Nutrition Laboratory, in the execution of this project.

LITERATURE CITED

BENJAMIN, H. 1951 Endocrine gerontotherapy. The use of steroid hormone combinations in male patients. J. Ins. Med., *6:* 3.

BROBECK, J. H. 1948 Food intake as a mechanism of temperature regulation. Yale J. Biol., *20:* 545.

CARTER, A. C., E. J. COHEN AND E. SHORR 1947 The use of androgens in woman. Vitamins and Hormones, *V:* 317.

EMMENS, C. W., AND A. S. PARKES 1947 Effect of exogenous estrogens on the male mammal. Ibid., *V:* 233.

GOLDZIEHER, J. W. 1949 The direct effect of steroids on the senile human skin. J. Gerontology, *4:* 104.

HELLER, C. G., AND W. O. MADDOCK 1947 The clinical uses of testosterone in the male. Vitamins and Hormones, *V:* 393.

HOOKER, C. W., AND C. A. PFEIFFER 1943 Effects of sex hormones upon body growth, skin, hair and sebaceous glands in the rat. Endocrinology, *32:* 69.

INGLE, D. J., AND B. L. BAKER 1951 The inhibition of hair growth by estrogens as related to adrenal cortical function in the male rat. Ibid., *48:* 764.

KOCHAKIAN, C. D. 1946 The protein anabolic effects of steroid hormones. Vitamins and Hormones, *IV:* 255.

KOCHAKIAN, C. D., E. ROBERTSON AND M. N. BARTLETT 1950 Sites and nature of protein anabolism stimulated by testosterone propionate in the rat. Am. J. Physiol., *163:* 332.

KORENCHEVSKY, V., AND M. DENNISON 1934 The effects of oestrone on normal and castrated male rats. Biochem. J., *28:* 1474.

LIGHT, A. E., J. A. TORNABEN AND E. J. DE BEER 1952 A comparison of the renal effects of polymyxin A, Aerosporin and a mercurial diuretic. Antibiotics and Chemotherapy, *2:* 63.

LINKSWILER, H., M. S. REYNOLDS AND C. A. BAUMANN 1952 Factors affecting proteinuria in the rat. Am. J. Physiol., *168:* 504

MEITES, J. 1952 Changes in nutritional requirements accompanying marked changes in hormone levels. Metabolism, *1:* 58.

MOORE, C. R., J. K. LA MAR AND N. BECK 1938 Cutaneous absorption of sex hormones. J. Am. Med. Assn., *111:* 11.

SELLERS, A. L., H. C. GOODMAN, J. MARMORSTON AND M. SMITH 1950 Sex difference in proteinuria in the rat. Am. J. Physiol., *163:* 662.

SELYE, H. 1950 The Physiology and Pathology of Exposure to Stress. Acta, Inc., Montreal, Canada.

SNEDECOR, G. W. 1946 Statistical Methods. The Iowa State College Press, Ames, 4th ed.

ZONDEK, B. 1935 Perkutane follikelhormontherapie. Schweiz. med. Wschr., *65:* 1168.

PLATE 1

EXPLANATION OF FIGURES

1 (Androgen-treated) shows the spleen with diffuse lymphatic tissue and an increase in red pulp.

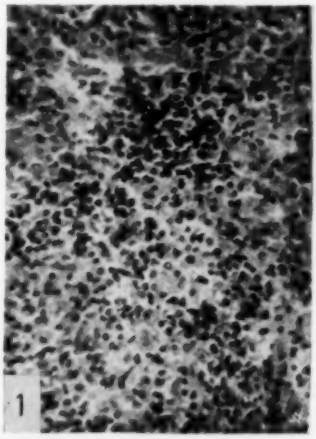
2 (Estrogen-treated) shows a well-defined splenic follicle. (Hematoxylin and eosin, × 210.)

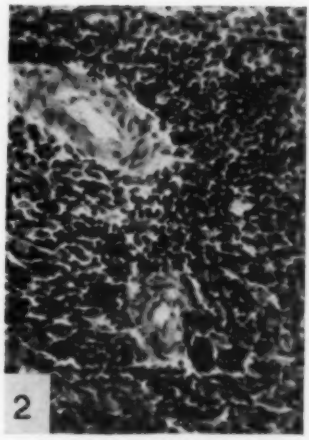
3 (Androgen-treated) shows the adrenal cortex with normal zona glomerulosa and zona fasciculata. There is a very slight lipoid depletion in the fasciculata.

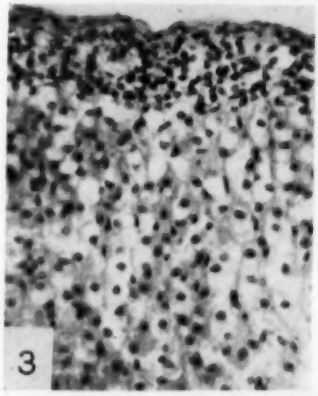
4 (Estrogen-treated) shows the adrenal cortex with a thickened capsule, and an ill-defined glomerulosa in which the cells are atrophic and the nuclei are predominantly pyknotic. There is a pronounced deposition of lipoid matter in the fasciculata, as seen by increased vacuolization. The sinusoids are dilated. (Hematoxylin and eosin, × 210.)

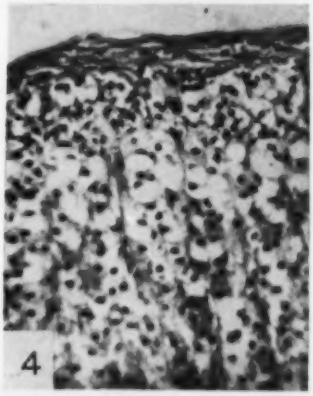
5 (Androgen-treated) shows testicular tissue in all stages of spermatogenesis.

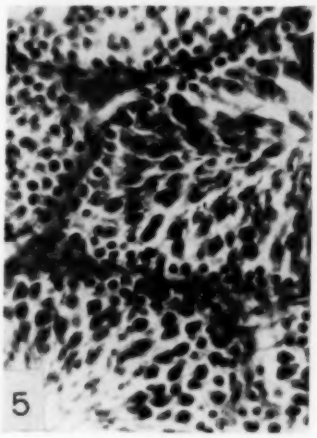
6 (Estrogen-treated) shows testicular tissue with grossly atrophic seminiferous tubules and a complete arrest of spermatogenesis at the spermatogonia level. The spermatogonia seen are subnormal. This tissue is non-functional. (Hematoxylin and eosin, × 210.)


1


2


3


4


5

6

FOLIC ACID DEFICIENCY IN THE DUCK [1]

O. NEAL MILLER,[2] J. W. GODDARD, ROBERT E. OLSON [3]
AND F. J. STARE

Department of Nutrition, Harvard School of Public Health, and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

TEN FIGURES

(Received for publication July 11, 1952)

INTRODUCTION

Although folic acid deficiency has been produced in a variety of animals and bacteria (Jukes and Stokstad, '48) no one has studied this deficiency in the duckling. Cardiac muscle metabolism as influenced by deficiencies of the B vitamins has been an important problem in this laboratory. It seemed of interest to include folic acid deficiency in this series of studies. This paper is devoted to the gross characterization of folic acid deficiency in the duckling, which resembles that found in other fowl (Jukes and Stokstad, '48). This deficiency in the duck is characterized by reduced growth rate and food consumption, macrocytic anemia, enlarged liver, and a lowered titer of trypsin and amylase activity in the duodenal contents. Marked histopathological changes were

[1] Supported in part by grants-in-aid from the Life Insurance Medical Research Fund, New York, N. Y.; Swift and Co., Chicago, Ill.; and the Nutrition Foundation, New York, N. Y.

[2] Part of the data is from a thesis submitted to the Faculty of the Graduate School of Arts and Sciences, Harvard University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana.

[3] This work was accomplished during the tenure of a research fellowship of the American Heart Association. Present address: University of Pittsburgh School of Public Health, Pittsburgh, Pa.

observed in the pancreas. A preliminary report on these results has been made (Miller, Goddard and Olson, '50).

EXPERIMENTAL

Newly hatched white Pekin ducklings were placed in heated raised-bottom cages and fed a purified diet (Hegsted and Perry, '47) for 4 or 5 days, at which time they averaged 100 gm in weight. They were then divided into control and experimental groups; the latter were fed a diet deficient in folic acid (but otherwise adequate) supplemented with 0.5% sulfaguanidine. Daily food consumption and growth records were kept on all birds. Determinations of hemoglobin, hematocrit values and red cell counts were made on all bloods from folic acid-deficient animals and their controls, and respiration studies were made on washed cells in a manner described by Miller, Olson and Stare ('49). Some of the livers from normal and folic acid-deficient ducks were analyzed for chloroform-extractable lipids, glycogen, total nitrogen and moisture. Amylase and trypsin titers of the duodenal contents were determined by standard methods (Todd and Sanford, '41). The folic acid content of the heart was determined microbiologically with a medium described by Capps, Hobbs and Fox ('48), using the organism *S. faecalis R.* The samples of tissue were predigested with folic acid conjugase, "hog kidney enzyme," of Bird et al. ('45). "Glucose" (reducing substances) was determined by the method of Reinicke ('42).

Specimens of heart, liver, small intestines, and pancreas from pair-fed and deficient birds were fixed in Zenker's and stained with Masson's trichrome stain, Mallory's Phloxine-Methylene Blue or Mallory's phosphotungstic acid hematoxylin. Paraffin sections were stained by Feulgen's technique for identification of desoxyribonucleic acid. Histochemical estimation of alkaline phosphatase (pH 9.5; Gomori, '39) was made on tissues fixed with chilled anhydrous acetone or 80% alcohol, using beta glycerol phosphate, fructose diphosphate, adenylic acid and glycose 6-phosphate for substrates.

RESULTS

Growth rates of folic acid-deficient, pair-fed control and normal control animals are shown in figure 1. During 10 to 14 days on the experimental diet, the food intake of the ex-

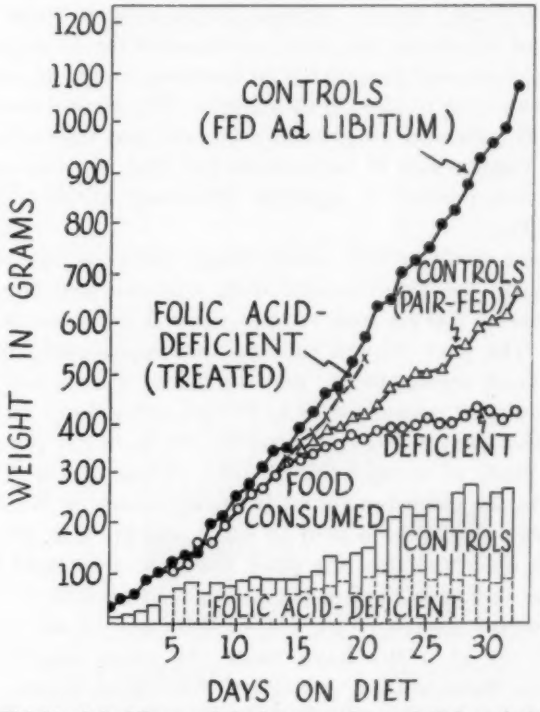


Fig. 1 Weight of ducklings and food consumed are plotted against length of experiment (days) for controls (fed ad libitum and pair-fed), folic acid-deficient ducklings and those treated by intraperitoneal administration of the vitamin.

perimental animals was approximately equal to that of normal birds; at this point the growth rate of deficient birds began to decline, and by the end of 28 days on the experimental diet, deficient birds weighed about 425, the pair-fed controls 675 and the normal controls about 1,100 gm. The deficient bird, in addition to being grossly smaller, has an

unkempt, greasy appearance which is characteristic of all birds that survive 28 days on the experimental diet.

Attempts to cure the deficiency syndrome were made with daily intraperitoneal injections of 1 mg of folic acid per bird, or with daily injections of 1 μg of vitamin B_{12} per bird (treatment lasted for 7 days). Anemic birds, used in these experiments, had consumed the experimental diet for 10 days. Birds receiving folic acid responded to treatment with an increased growth rate and remission of anemia. The birds treated with vitamin B_{12} showed no growth response and no remission of anemia, from which it was concluded that the experimental diet did not produce a multiple deficiency of folic acid and vitamin B_{12}.

Deficient birds gained about 50 gm between the 18th and 28th day on the experimental diet, whereas pair-fed control ducks gained 200 gm and normal control birds gained about 400 gm. The poor growth rate was accompanied by less than normal food consumption, and diarrhea. Ducks fed the experimental diet consumed 70 to 100 gm of food per day, which is enough to support rapid growth (30 to 40 gm per day) in normal birds of comparable weight. These findings appear similar to the situation found in sprue, in which Darby et al. ('46) have shown folic acid to be of therapeutic value.

It was observed that the liver from the folic acid-deficient duck was enlarged, 5.67 ± 0.21 compared to $4.58 \pm 0.28\%$ of body weight. Keith et al. ('48), Williams et al. ('49) and Schaefer et al. ('50) have made the same observation in chicks on diets with a restricted folic acid intake. Livers from animals that survived 28 days on the experimental diet were analyzed for total solids, total nitrogen, fat and glycogen and compared with those of their normal controls. Figure 2 shows the partition of livers from folic acid-deficient and normal birds. The liver of the deficient bird is characterized by its low glycogen, high fat and high protein (total nitrogen) content.

If folic acid deficiency as produced in the duck is similar to sprue, one might expect a low blood sugar concentration

and a low content of liver glycogen due to faulty absorption. It has been reported (Williams et al., '49) that folic acid-deficient chicks have a high concentration of blood sugar. Blood samples from two groups of deficient birds were analyzed for glucose (reducing substance) and found to be no

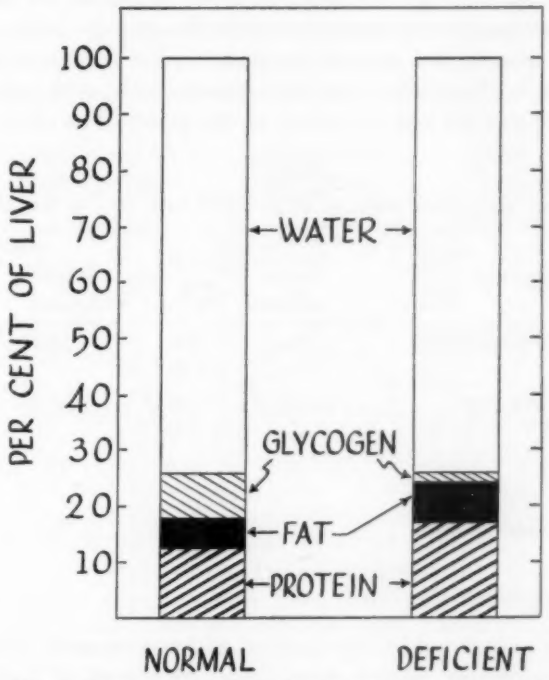


Fig. 2 Shows the proportion of water, glycogen, fat, and protein in livers from normal and folic acid-deficient birds.

different from samples from normal control birds (table 1). The ducks in these experiments had free access to food. Our results are not in agreement with the findings of Williams et al.

Normal ducklings are usually anemic when hatched. If they are allowed to grow on an adequate control diet, the red cell count gradually rises to about 2.5 million per cubic millimeter after 10 to 12 days. When ducks were fed the ex-

perimental diet, the red cell count declined after the 4th day and continued to drop for 12 to 15 days (fig. 3). A gradual increase in blood hemoglobin was observed in normal birds for about 10 days, but a less rapid rise was observed in deficient animals. Between the 10th and 12th day, the blood hemoglobin in deficient birds began to decline (fig. 3). This change was consistent with the change from normocytic to macrocytic anemia, as shown also in figure 3.

Figure 4 shows the relation between folic acid content of the heart and the red cell count of the blood. The slope of the

TABLE 1

The effect of folic acid deficiency on the blood sugar level in the fed duck

OBSERVATION	DEFICIENT		NORMAL	
	Number of animals	Mean value	Number of animals	Mean value
Blood sugar concentration (mg %)	10	322 ± 24 [1]	10	291 ± 14
Red cell count × 10^6 per mm^3	10	1.79 ± 0.12	10	2.36 ± 0.09
Hematocrit	10	27.6 ± 0.4	10	33.1 ± 0.6
Mean corpuscular volume	10	154 ± 7	10	141 ± 2

[1] Standard error of the mean.

line was calculated by the method of least squares and found to be significant, with a correlation coefficient of 0.51 and a probability of error of less than 5%. Normal heart muscle contained 268 ± 14 mμg per gram fresh weight, which is nearly the same value reported by Mitchell and Isbell ('42) for rat heart. Deficient cardiac tissue contains 145 ± 14 mμg per gram fresh weight.

Blood cells from deficient and normal ducks were allowed to respire under the same experimental conditions used in studies of heart muscle respiration (Miller, Olson and Stare, '49). The effect of folic acid deficiency on respiration and

pyruvate utilization of washed blood cells is shown in table 2. During the initial phase of the deficiency, there was a significant depression of oxygen uptake and of pyruvate utilization. With the onset of macrocytic anemia, however, beginning the 11th or 12th day, pyruvate utilization and oxygen uptake returned to normal.

It has been demonstrated that folic acid-deficient birds cannot utilize their food as efficiently as pair-fed control animals. This finding prompted experiments which might give some

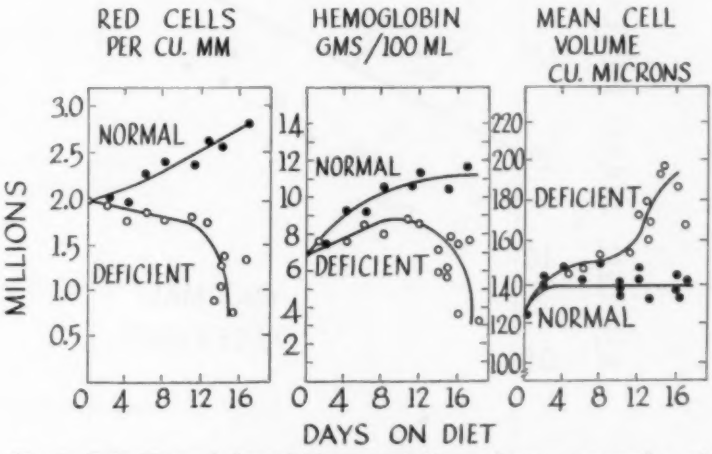


Fig. 3 Red cell count, hemoglobin concentration and mean corpuscular volume are plotted against length of experiment (days). Values for both normal and folic acid-deficient birds are shown.

insight into the underlying cause. Both amylase and trypsin activity were found to be depressed by 85% in the duodenal contents of folic acid-deficient ducks, which is not the case in sprue. After an experimental period of 28 days, specimens of liver, pancreas, intestine and heart were taken from deficient and normal birds for histological examination.

Results of histological examination of the various tissues are shown in figures 5 through 10 (plate 1). Liver cells in sections from the experimental birds contain fewer glycogen

vacuoles, increased cytoplasmic ground substances (possibly protein, in view of the increased nitrogen content of the liver), less chromatin in the nuclei, and reduced alkaline phosphatase activity with adenylic acid (muscle) as substrate.

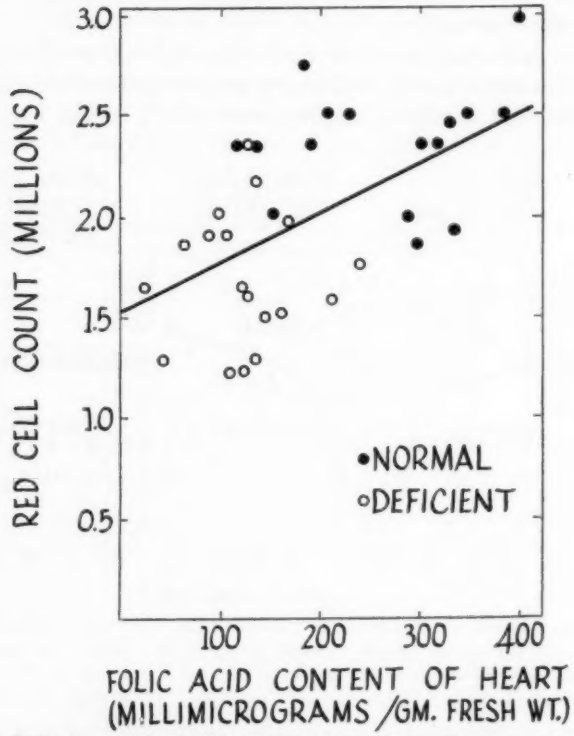


Fig. 4 Red cell count is plotted against folic acid concentration in the hearts of both normal and folic acid-deficient ducklings.

Similar results were obtained when glycerol phosphate, glucose 6-phosphate, or hexose diphosphate was used as substrate for phosphatase action. Histological changes in the pancreas from the experimental bird showed reduced desoxyribonucleic acid content of their nuclei, and abnormal mitochondria with respect to agglomeration of zymogen grains with

TABLE 2

The effect of folic acid deficiency on respiration and pyruvate utilization in washed blood cells from the duck in vitro

	DEFICIENT			NORMAL	
	Days on diet	Number of animals	Mean value	Number of animals	Mean value
Mean corpuscular volume of cells	8	6	139 [1] ± 7 [2]		
	13	5	167 ± 8	11	140 ± 6
Oxygen uptake	8	6	49 ± 6		
	13	5	90 ± 11	11	68 ± 4
Pyruvate uptake (pyruvate 5 mM/L)	8	6	51 ± 7		
	13	5	80 ± 7	11	77 ± 6
Lactate from pyruvate	8	6	24 ± 4		
	13	5	31 ± 2	11	30 ± 2
Net pyruvate uptake	8	6	27 ± 7		
	13	5	49 ± 7	11	47 ± 6

[1] All respiratory data represent microliters per milliliter of standard blood (hematocrit, 38.0).

[2] Standard error of the mean.

no polar orientation. This suggests that cells deficient in folic acid are unable to release the zymogen that is normally formed. Sections of cardiac muscle from the deficient birds gave evidence of degeneration as indicated by varying degrees of granularity. Virtually no changes were observed in the intestine.

DISCUSSION

It is clear that folic acid is essential for good nutrition of the duck. Although a deficiency of other B vitamins has been shown to affect the growth rate after two or three days on an experimental diet, 10 or 12 days were required in these experiments to show a depression in growth rate. The administration of folic acid to the deficient birds prevents any change in growth or in the blood picture. The intraperitoneal administration of vitamin B_{12}, however, to folic acid-deficient birds causes no growth response or remission of anemia. These data are in contrast to those reported by Dietrick et al. ('49) and Schaefer et al. ('50) with respect to chicks. These workers postulated an interrelationship between folic acid and vitamin B_{12}. Luckey et al. ('46) have shown that the addition of sulfasuxidine to the diet of the chick increases the folic acid requirement of the chick by three-fold. In view of this finding, it seems possible that the administration of vitamin B_{12} might stimulate the synthesis of folic acid by the intestinal flora.

It is of interest that the folic acid-deficient duck shows a diminished content of glycogen in the liver. In view of the fact that alkaline phosphatase activity (pH 9.5) is depressed in liver tissue from deficient birds, it is suggested that this may in part account for the low glycogen content. Recently Meyerhof and Green ('50) have shown that intestinal alkaline phosphatase is capable of catalyzing phosphorylation reactions if the bond energy of the phosphate donor is higher than that of the phosphate acceptor.

Prusoff et al. ('48) have shown that bacteria *(L. casei)* grown on a medium with suboptimum levels of folic acid produce less desoxyribonucleic acids. Data presented here support their findings, in that liver and pancreas from folic acid-deficient birds showed less chromatin (desoxyribonucleic acid) in their nuclei. It is conceivable that the primary defect in folic acid deficiency in the duckling is a failure of phosphorylation to occur in both carbohydrate and nucleoprotein metabolism.

SUMMARY

Folic acid deficiency has been produced in ducklings and is characterized by reduced growth rate, only slightly reduced food consumption, macrocytic anemia, enlarged liver, and a lowered titer of trypsin and amylase activity in the duodenal contents. The liver from the deficient bird is low in glycogen and slightly high in fat and in total nitrogen content. Blood glucose is normal. Under the experimental conditions employed, no interrelationship was found between folic acid and vitamin B_{12}.

Histopathological examination of deficient tissue showed absence of the secretory cycle of zymogen granules in the pancreas, decreased alkaline phosphatase in the liver, and blurring of striation in the heart.

LITERATURE CITED

BIRD, O. D., B. GRESSLER, R. A. BROWN, C. J. CAMPBELL AND A. D. EMMETT 1945 The microbiological assay of vitamin Bc conjugate. J. Biol. Chem., *159:* 631.

CAPPS, B. F., N. H. H. HOBBS AND S. H. FOX 1948 A dehydrated experimental medium for the microbiological assay of folic acid. J. Bact., *55:* 689.

DARBY, W. J., M. E. CHERRINGTON AND J. M. RUFFIN 1946 Plasma level of tocopherol in sprue. Proc. Soc. Exp. Biol. Med., *63:* 310.

DIETRICK, L. S., C. A. NICHOL, W. J. MONSON AND C. A. ELVEHJEM 1949 Observations on the interrelation of vitamin B_{12}, folic acid and vitamin C in the chick. J. Biol. Chem., *181:* 915.

GOMORI, G. 1939 Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exp. Biol. Med., *43:* 23.

HEGSTED, D. M., AND R. L. PERRY 1947 Nutritional studies with the duck. V. Riboflavin and pantothenic acid requirements. J. Nutrition, *35:* 411.

JUKES, T. H., AND E. R. L. STOKSTAD 1948 Pteroylglutamic acid and related compounds. Physiol. Rev., *28:* 51.

KEITH, C. K., W. J. BROACH, D. WARREN, P. L. DAY AND J. R. TOTTER 1948 Xanthine oxidase and tyrosinase in the livers of chicks receiving graded levels of dietary pteroylglutamic acid. J. Biol. Chem., *176:* 1095.

LUCKEY, T. D., P. R. MOORE, C. A. ELVEHJEM AND E. B. HART 1946 The activity of synthetic folic acid in purified rations for the chick. Science, *103:* 682.

MEYERHOF, O., AND H. GREEN 1950 Synthetic action of phosphatase. II. Transphosphorylation by alkaline phosphatase in the absence of nucleotides. J. Biol. Chem., *183:* 377.

MILLER, O. N., J. W. GODDARD AND R. E. OLSON 1950 Physiological and histological changes in the folic acid deficient duckling. Fed. Proc., *9:* 366.

MILLER, O. N., R. E. OLSON AND F. J. STARE 1949 Folic acid deficiency and the respiration and pyruvate utilization of duckling erythrocytes and cardiac muscle. Ibid., *8:* 390.

MITCHELL, H. K., AND E. R. ISBELL 1942 Vitamin Content of Tissues. University of Texas Publications, No. 4237, p. 37.

PRUSOFF, W. H., L. J. TEPLY AND C. G. KING 1948 The influence of pteroylglutamic acid on nucleic acid synthesis in *L. casei*. J. Biol. Chem., *176:* 1309.

REINICKE, R. M. 1942 The determination of glucose in minimal quantities of blood. Ibid., *143:* 351.

SCHAEFER, A. E., W. D. SALMON, D. R. STRENGTH AND D. H. COPELAND 1950 Interrelationship of folacin, vitamin B_{12} and choline. J. Nutrition, *40:* 95.

TODD, J. C., AND A. H. SANFORD 1941 Clinical Diagnosis by Laboratory Methods. W. B. Saunders Co., Philadelphia, Pa., 9th ed., p. 405.

WILLIAMS, J. N., JR., C. A. NICHOL AND C. A. ELVEHJEM 1949 Relation of dietary folic acid and vitamin B_{12} to enzyme activity in the chick. J. Biol. Chem., *180:* 689.

PLATE 1

EXPLANATION OF FIGURES

5–10 In figures 5, 6 and 8 the sections were fixed in Zenker's and stained with Masson's trichrome stain. The section shown in figure 7 was fixed in Zenker's and stained with Mallory's phosphotungstic acid and hematoxylin. Sections in figures 9 and 10 were fixed in 80% alcohol and treated by the Gomori technique for alkaline phosphatase. All sections are 360 magnification.

5 Liver from deficient bird shows very small vacuoles (glycogen dissolved during fixation). Cytoplasm is darkly stained.

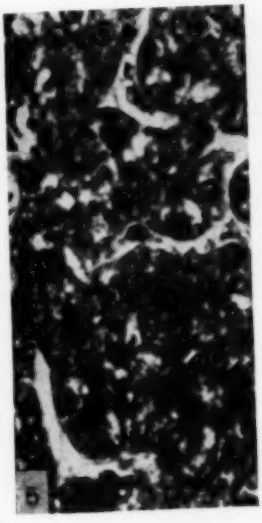
6 Small intestine from a deficient bird appears to be within normal limits.

7 Cardiac muscle from a deficient bird shows blurred striation and increased granularity.

8 Pancreas from a deficient animal which shows loss of acinar orientation and gives evidence of degeneration and uneven staining of the cytoplasm with pale vesicular nuclei.

9 A section of liver from a normal bird (pair-fed) which demonstrates alkaline phosphatase activity in the hepatic sinusoids and bile capillaries.

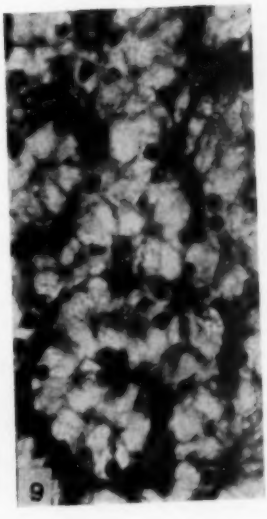
10 A section of liver from a folic acid-deficient bird which was prepared at the same time as that shown in figure 9 using the same technique. Markedly less phosphatase activity is demonstrated.

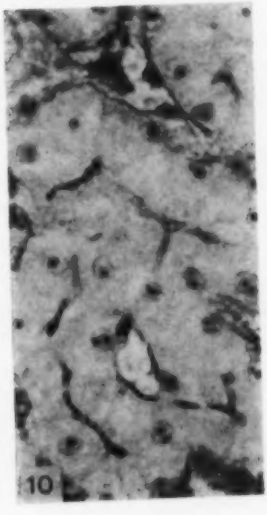

5

6

7

8


9


10

ZINC DEFICIENCY IN SUCKLING MICE DEPRIVED OF COLOSTRUM

HIDEO NISHIMURA

Anatomical Institute, Medical Faculty of Kyoto University, Yoshidakonoecho, Sakyo-ku, Kyoto, Japan

ELEVEN FIGURES

(Received for publication June 16, 1952)

It is generally recognized that the mammary secretion in various mammals is peculiarly well adapted to meet the nutritive demands of early postnatal life for each particular species. Bottle-fed infants are thought to be less resistant to disease than breast-fed infants. Anemia has been observed in infants and young animals fed goat's milk (Rominger and Bomskov, '35; Orten and Smith, '36; Takuma, '41). Furthermore, cow's milk is said to be inadequate for the growth and vigor of young pigs (Bunger and Blocker, '30) and for fertility in rats (Daniels and Hutton, '25). It has also been reported (Parkes, '29) that young mice suckled by rats show abnormal growth. Colostrum differs considerably from mature milk with respect to its content of proteins, inorganic salts, enzymes, immune bodies and vitamins. Smith and Little ('22) reported that newborn calves deprived of colostrum show a higher mortality and greater incidence of colitis than breast-fed calves, suggesting that the colostrum contains important antibodies. These facts led me to undertake the studies reported here, in which newborn mice were nursed by foster-mothers in post-parturitional stages of lactation. Observations were made concerning the effects on growth and development of the young and on the morphogenesis and histogenesis of certain organs and tissues.

METHODS

For this study about 1000 newborn mice were used, representing 200 litters delivered by mothers of the stock colony fed a diet consisting of crushed rice, bean powder, embryo buds of rice, dried fish, yeasts, salts, calcium carbonate and vegetables. Members of each litter were about equally divided into two groups within 12 hours after birth. The mice of group 1 were left with their own mothers as controls. Those of group 2 were placed for 13 to 20 days post partum with foster mothers whose own pups had been removed. In all cases the foster mother eagerly reared the foster pups. Thus, the two groups differed only in regard to the milk received. On alternate days, during an experimental period of three weeks, the body weight, body-length and tail-length were measured and external developmental changes such as opening of the auricula and eyelids and eruption of the incisors were recorded. The young were also examined for any abnormalities of the skin, hair, nails, eyes, oral cavity, feces, urine, respiratory functions and motor coordination. The state of ossification in the caudal vertebrae was determined, using the potash-glycerin method with alizarin staining (Lundvall, '27). For histologic study, paraffin and celloidin sections of formalin-fixed organs and tissues were routinely stained with hematoxylin and eosin; specialized procedures employed included Best's carmine for liver glycogen, methyl green-pyronin (Unna-Pappenheim) for mast cells, Gomori's chrome alum-hematoxylin-phloxine stain for islet cells of the pancreas and Heidenhain's iron hematoxylin after Carnoy's fixative for counts of mitotic figures. Blood from the tail vein was used for differential counts, study of stained films and hemoglobin determinations. Certain special procedures and chemical analyses carried out are indicated later.

RESULTS

Growth and differentiation

Growth of the foster pups, as measured by body weight, was moderately good but usually somewhat inferior to that of the controls (fig. 1). The data on nose-anus length and tail

length revealed similar differences between the two groups. It is apparent that lactation in the mouse can be continued until about the 35th day after parturition by the act of suckling. In order to obtain further data on lactation performance, daily weight gains were recorded for 6 litters reared to 15 to 19 days of lactation by their own mothers, and for 6 foster litters of newborn pups given to these same mothers to rear for a comparable period. The pattern of daily weight gain (fig. 2) was much the same in the real and foster litters, showing an early rise and a later decline; how-

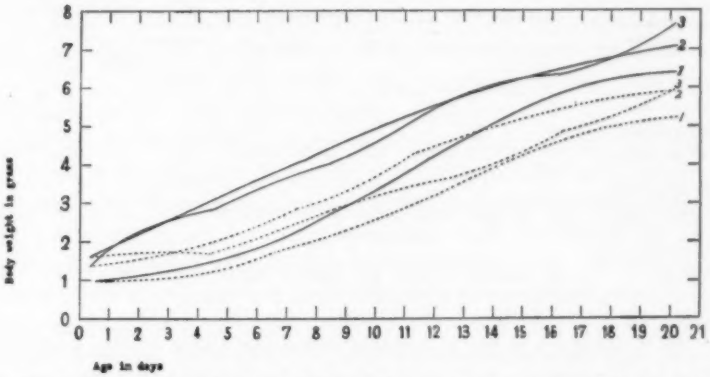


Fig. 1 Average growth curve of three litters each, of normal and foster sucklings. ——— Normal sucklings. - - - - Foster sucklings. The same number is always used for the same litter.

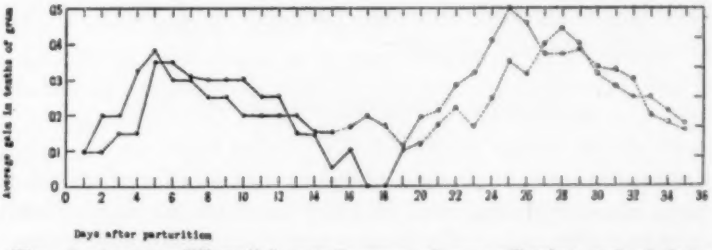


Fig. 2 Average differential growth curve of own offspring and of foster sucklings reared by the same mother (two cases). ——— Own sucklings. - - - - Foster sucklings.

ever, the peak of growth increment appeared a little later in the foster than in the real litters. As a corollary to Enzmann's ('33) conclusion that the differential growth curve parallels the milk production curve of mice, the above findings indicate that milk production is dependent chiefly on the suckling capacity of the young.

TABLE 1

Effects of suckling by foster mothers upon development of external ear, eruption of lower incisors and separation of eyelids

DEVELOPMENTAL PROCESS	CONTROL PUPS		FOSTER PUPS		NO. OF LITTERS	FOSTER PUPS COMPARED TO EACH CONTROL		
	No.	Mean age	No.	Mean age		Accelerated	Delayed	Same
						%	%	%
Separation of ear auricula	39	4.18 ± 0.097	45	4.42 ± 0.091	39	5	36	59
Eruption of lower incisors	45	11.42 ± 0.195	36	10.41 ± 0.189	36	50	17	33
Separation of eyelids	44	15.83 ± 0.355	20	13.72 ± 0.255	20	45	10	45

In the foster pups postnatal differentiation of the external ear was normal; yet there was a tendency for eruption of incisors and dysjunction of the eyelids to be accelerated (table 1), despite the fact that body growth was generally inferior to that of control pups. Ossification of caudal vertebrae was somewhat retarded, probably as a result of their generally retarded growth.

Changes in external appearance and general behavior

Beginning about the end of the first week of age, the foster pups showed retardation of hair development and peculiar changes of the skin. There was marked thinning of the hair, or often complete alopecia, over the dorsal thoracic and lumbosacral regions, often extending to the proximal part of

the tail and extensor surfaces of the forearm and leg (fig. 3). However, the vibrissae in the region of the upper lip, nose and eye remained essentially normal. The skin of the dorsum became glossy, wrinkled and thickened (fig. 3). Occasionally the auricula of the ear was thickened. Subsequently, scaling occurred over the posterior dorsum, later extending over the proximal portion of the extremities, the caudal abdomen and head. Usually, this exfoliation continued for two or three days. Inflammatory signs were absent. Occasionally the mobility of the hind extremities, with the thickened skin, was so disturbed that the animal slowly rotated around a fixed foot like the hand of a clock. When these lesions were severe, the animals died in the stage of marked scaling and showed ecchymosis at the end of the tail, the fingers, toes and the auricula of the ear. Occasionally, at 14 to 18 days of age, localized swellings were observed in the more distal portions of these appendages; these appeared frequently as a rosary-like tail (fig. 4) and less frequently as clubbed digits or thickened ear auricula. In most cases the nails exhibited an abnormally pronounced plantar curvature (fig. 5). In animals showing milder lesions there was at first retarded growth of hair and slight glossiness of the skin over the middle zone of the dorsum, followed by sparse distribution of hair over the whole body.

Histological studies

In confirmation of macroscopic findings, pronounced skin lesions were found in the foster pups. The epidermis was nearly three to 4 times its normal thickness (figs. 6 and 7). There was excessive superficial desquamation of cells, due to hyperkeratinization. The stratum germinativum was composed of 5 to 6 cell layers, instead of the usual two or three layers, and showed evidence of marked increase in mitotic proliferation; the frequency of mitotic cells in the stratum germinativum of the dorsal skin of three control and three foster pups, based on examination of between 5,185 and 6,756 cells per animal was 0.18% and 0.78%, respectively. The corium

also exhibited increased thickness. The subcutaneous layer, however, was atrophic and subcutaneous fat was sparse or absent. In sections stained with methyl green-pyronin (Unna-Pappenheim) the mast cells in the corium and the subcutaneous layer were considerably more numerous than in control pups. This was also true of macrophages as revealed by subcutaneous injections of Trypan blue. Microscopically the hair shaft was short, slender and irregular in thickness; its medullary substance was poorly developed, being composed of a row of irregularly shaped and loosely arranged polyhedral cells. The hair follicles were smaller than normal, and their distribution was sparse and irregular. Sebaceous glands were normal in structure.

After the second week of lactation there was continued thickening of the epidermis and hypoplasia of the hairs. Although the epidermis became thicker than before, and the stratum germinativum was composed of 7 to 8 cell layers, there was no crust of hyperkeratinized cells. Hypoplasia of the medullary substance and irregular and sparse arrangement of the hair follicles were evident. The rosary-like tail was characterized by remarkable thickening of the epidermis, much pyknosis and karyorrhexis of degenerating cells, sparse distribution of the hairs with little medullary substance, and congestion of the capillaries throughout the skin and subcutaneous layer (figs. 8 and 9). There was also some hyperplasia of the hood-like epithelial ingrowth of the nipples.

The following tissues and organs were examined histologically: oral mucosa, tongue, salivary glands, alimentary canal, liver, pancreas, nasal mucosa, trachea, lungs, heart, larger blood vessels, bone marrow, spleen, cervical and mesenteric lymph nodes, various portions of the genito-urinary system of both sexes, ductless glands, striated muscles, brain, spinal cord, peripheral nerves, long and flat bones, articular cartilages and the ocular apparatus. The following alterations were noted: the esophageal epithelium showed localized areas of superficial desquamation of epithelial cells; there was moderate congestion of the capillary vessels in the liver and

lung, moderate congestion and reduction of follicles in the spleen; in the palpebral conjunctiva the overlying layer became squamous and occasionally the stratified cuboidal or columnar epithelium was transformed into a stratified squamous epithelium (figs. 10 and 11). Otherwise, all the structures referred to above could be considered histologically normal.

Differential blood cell studies were made on 4 control normal and 4 foster sucklings reared by their own and by foster mothers for 10 to 13 days. Smears of blood taken from the tip of the tail and stained with Giemsa's solution revealed no significant difference between the two groups; the same was true of total erythrocyte and leukocyte counts and hemoglobin levels, which averaged, respectively, 6.5 millions, 5,800 and 74% for the control pups and 6.1 millions, 5,400 and 71% for the foster pups.

Prevention of the cutaneous lesions

In order to investigate the genesis of the cutaneous lesions, the following studies were carried out.

Relation of lesions to stage of lactation of foster mothers. To study the influence of the stage of lactation of foster mothers upon the occurrence of the characteristic skin lesions, 152 newborn litters were allowed to be nursed by foster mothers at zero to 32 days after parturition. Reference to table 2 shows that the young fostered by mothers which had nursed their own litters for no more than one day had no lesions; nearly one-half of those fostered by mothers which had lactated 5 to 10 days had moderate lesions; as the postpartum age of the foster mother increased there was a corresponding increase in frequency and severity of the lesions. Litters reared by mothers zero to 10 days postpartum grew as well as the controls, but those nursed by mothers in later stages of lactation showed growth retardation which generally paralleled the intensity of the cutaneous lesions. Members of the same litter usually responded rather uniformly. The young which were placed with mothers 23 days or more postpartum rarely survived the lactation period. If they did

survive they were quite healthy. One pup was successfully nursed by a foster mother beginning at 32 days after parturition.

Effect of alternate nursing by own mother and foster mother. In one experiment, 7 newborn mice suckled by their own mothers for 2.5 to 4 days before transfer to foster mothers (13 to 18 days postpartum) were normal; 8 of 9 others suckled for periods of only 0.5 to 1.5 days before transfer developed

TABLE 2

Occurrence of skin alterations in relation to post-parturitional stage of the foster mothers

DAY STAGE OF THE FOSTER MOTHERS	NO. OF LITTERS	TOTAL NO. OF SUCKLINGS	CLASSIFICATION OF THE CASES ACCORDING TO OCCURRENCE OR DEGREE OF THE SKIN ALTERATIONS			
			1	2	3	4
0	3	12	0	0	0	12
1	2	6	0	0	0	6
5– 7	4	8	0	4	0	4
8–10	8	20	0	2	9	9
11–13	19	61	26	15	9	11
14–16	61	214	151	40	13	10
17–19	41	115	104	9	0	2
20–22	8	20	18	0	0	2
23–32	6	17	14 (died)	0	0	3

[1] Severe changes showing alopecia, pronounced desquamation of the epidermis and a rosary-like tail.

[2] Moderate changes showing sparse hair and glossy skin over the dorsal body region.

[3] Very slight changes, characterized by retarded hair growth over the dorsum.

[4] No alterations.

skin lesions. Members of the same litters placed with the foster mothers at birth consistently exhibited pronounced skin alterations.

In another experiment, 18 newborn mice were first placed with foster mothers (13 to 18 days postpartum) and, after periods of two to 5.5 days, were returned to their own mothers. Foster nursing of the newborn for more than three days led to skin lesions in 11 of 12 mice, the lesions increasing in severity with increased duration of the foster nursing period.

Members of the same litters left with the foster mothers always showed advanced skin lesions.

Lastly, a combination of both ways of nursing was carried out. Four suckling mice were allowed to nurse their own mother and the foster mother alternately, for 12-hour periods. Skin lesions were severe in two cases, slight in one case and absent in the other animal.

The results of these experiments demonstrate that the mother's milk during the first few days after parturition provides some nutrient (or nutrients), not present or not provided in sufficient amounts in later milk, which is necessary for normal development of the newborn mouse.

Preventive experiments by administration of various nutrients. Assuming that the skin lesions were due to deficiency of something present in colostrum, various nutrients or substances known to influence the development or maintenance of the skin were administered to mice reared from birth by foster mothers (13 to 18 days postpartum) to determine their prophylactic value. In this study each litter was divided into two groups, one group being used to test the various substances and the other group being left as controls; whenever the latter failed to develop pronounced lesions, the test group was discarded. The substances tested but found ineffective included lactoflavin, nicotinic acid, vitamins C, B_6, A and D, casein, human milk, potassium iodide, magnesium sulphate, and injections of insulin and human and rat blood. Some amelioration of the disorders was obtained with vitamin B_1, with a commercial product [1] containing vitamin B_2 and phosphoric anhydride, with yeast emulsion and with an emulsion of rat liver. Effective protection was given by single doses of zinc chloride or zinc acetate (0.005 ml of a 0.05% solution, providing about 7 μg of zinc) given orally on the day of birth. These findings provide strong evidence that the disorders in the foster pups were due to zinc deficiency. The mitigating effect of liver pap and yeast may be attributed to the presence in them of a certain amount of zinc, as demonstrated quali-

[1] Wakaflavin.

tatively by spectroscopic analysis but not measured quantitatively. The effect of crystalline thiamine and of the commercial product mentioned above, which are presumably free of zinc, is more difficult to explain; one might question whether they have some sparing action on zinc metabolism.

It also seemed of interest to determine whether administration of inorganic zinc to the foster mother would influence the occurrence of skin lesions in the foster pups. It was found that providing foster mothers with a 0.1% zinc acetate solution instead of drinking water did not prevent skin alterations in the foster pups. This indicates that the mammary barrier, at least in the later phases of lactation, is relatively impermeable to inorganic zinc.

Chemical composition of foster and control pups

Suckling young 9 to 13 days of age reared by their own mothers (controls), and litter mates reared by foster mothers and showing pronounced skin lesions, were decapitated immediately, minced into pieces, and subjected to chemical analysis. The percentage of water was estimated from the loss of weight after heating the material at 105°C. until its weight became constant. The dry substance obtained was incinerated in a porcelain crucible and the ash thus obtained estimated. Furthermore, total nitrogen in the dry substance was determined by Pregl's micro-Kjeldahl method, and the raw fat estimated after extraction with ether in a Soxhlet apparatus. The zinc content of the ash was determined by spectrographic analysis.

The foster pups, when compared to control pups of the same age, showed a higher content of water and ash and an appreciably lower content of zinc (table 3); they also showed a slightly higher content of nitrogen and a somewhat lower content of extractable fat. These data are in accord with other evidence that a deficiency of zinc is primarily responsible for the characteristic skin lesions observed in young mice deprived of their mother's colostrum and reared by foster mothers in later stages of lactation. Efforts made to determine the zinc

content of the milk present in the stomach of the suckling mice immediately after nursing were not successful, due possibly to the difficulty of accurately measuring the small amounts of zinc present.

TABLE 3

Chemical analyses of control and litter-mate foster pups, 9 to 13 days of age

TYPE OF PUP	WATER	DRIED CARCASS				
		Fat	Total nitrogen	Total ash	Zinc	Zinc
	%	%	%	%	*mg/100 gm body wt.*	*mg/100 gm ash*
Control	71.6[5] *	29.3[4]	8.18[4]	8.56[3]	1.56[3]	70.6[3]
Foster	75.5[6]	27.9[4]	9.09[4]	9.31[3]	1.14[3]	51.7[3]

* Superscript numbers represent number of mice analyzed, on the basis of which the recorded average values were obtained.

DISCUSSION

It should be pointed out that a variety of lesions affecting the epidermis and its derivatives (hair follicles, sebaceous glands, sweat glands) have been observed in the rat and mouse following deprivation of vitamin A, essential fatty acids, riboflavin, pantothenic acid, vitamin B_6 and biotin (Follis, '48), and that alopecia, hyperkeratosis and atrophy of the epidermis have been described (Lippincott and Morris, '41, '42; Nielsen and Black, '44; Jones et al., '45) in mice deficient in various components of the vitamin B complex. Despite certain superficial resemblances, the symptoms and histopathologic changes observed in the foster suckling mice used in this study differ in many respects from the conditions described in the above-mentioned studies, and from those occurring in rats deficient in sodium (Orent-Keiles et al., '37) or magnesium (Sullivan and Evans, '44); on the other hand, they closely resemble the changes described in rats on diets low in zinc (Todd et al., '34; Stirn et al., '35; Day and McCollum, '40; Follis et al., '41). This is in keeping with the evidence provided by the prophylactic tests and the chemical analyses carried out on the control and foster pups in the present study.

In the studies of Follis et al. ('41), which provide a detailed account of the histopathology of zinc deficiency in the rat,

there was marked retardation of growth and, after several weeks, marked thickening and hyperkeratinization of the epithelial lining of the esophagus, and alterations in the skin quite similar to those described in the present study but restricted to the dorsum of the body. In a few instances there was vascularization and leukocytic infiltration of the cornea similar to that found in riboflavin deficiency. The only reported study of zinc deficiency in mice (Day, '42) mentions retarded growth and alopecia of the shoulders, back of the neck and part of the face, but makes no reference to histopathologic changes. Comparing the present findings in foster suckling mice with those of Follis et al. ('41), certain points of difference are apparent. The skin alterations appeared much earlier and often extended to the dorsum of the upper arm and thigh, and also to the tail, ears and distal portions of the extremities; there was more marked mitotic proliferation in the basal layer of the epidermis, but much less alteration of the esophageal epithelium; although the epithelium of the palpebral conjunctiva became squamous, there were no corneal changes. Undoubtedly, some of these differences are related to the earlier age, and consequently the less differentiated state of certain tissues and organs, at the time of onset of the deficiency state; there may also be species differences in response to the epithelial derivatives to zinc deficiency.

The experimental evidence presented here, relative to the higher content of zinc in colostrum than in later milk, raises the question as to whether this represents a compensation for a restricted placental transfer of this trace element. The recent findings of Spray and Widdowson ('50) indicate that the newborn of various laboratory animals, including the mouse, possess appreciable body stores of zinc, suggesting reasonably efficient placental transfer. My own observations that manifestations of zinc deficiency can occur in suckling mice denied access to colostrum further emphasize the importance of trace elements during early postnatal life and the provision made for such needs in the mammary secretions of different species of mammals. It is natural to assume that later milk provides

considerable zinc but often an insufficient amount if not supplemented by that of the colostrum, and that a considerable utilization of zinc occurs during the period of nursing. Such assumptions are in accord with the statements of Spray and Widdowson ('50) that during early phases of growth in the rat and in the rabbit there is a considerable increment in body stores of zinc, much of which becomes concentrated in epidermal structures where it presumably becomes attached to keratin proteins. They also report that zinc, which also accumulates in the liver and spleen, diminishes rather abruptly after weaning. Spray ('50) also comments upon the relatively high concentration of zinc in the rat's milk, and the fact that a considerable portion of it is probably used for the growth of hair by the suckling young.

Some differences were noted between the content of water, fat and ash in the body of deficient and control mice. It remains to be determined whether these are related to metabolic derangements resulting from zinc deficiency or to constitutional imbalance secondary to alopecia and thickening of the epidermis.

Lastly, the mitigating influence of vitamin B_1 upon the development of the disorders is of interest in connection with the metabolic role of zinc. Bertrand and Bhattacherjee ('35) indicate that in zinc-depleted rats the metabolic functions of vitamin B_1 are suppressed. Imada ('40), experimenting with rabbits, reports that there is a synergistic interaction between zinc and vitamin B_1 in the intermediary metabolism of carbohydrates. These facts seem to be in accord with the findings reported in this study.

SUMMARY

Newborn mice nursed by foster mothers in later stages of lactation (usually 13 to 18 days) developed specific disorders, the frequency and severity of which increased as the initial post-parturient stage of the foster mother increased. Newborn mice nursed by their own mothers for two and one-half to 4 days before transfer to foster mothers were normal; the

mice that were fed by foster mothers for 3.5 to 5.5 days and then returned to their mothers usually developed disorders.

The disorders consisted of retarded growth and ossification, accelerated eruption of incisors and separation of eyelids, alterations of the dorsal skin (alopecia, thickening and hyperkeratinization of the epidermis with no inflammatory signs, thickening of the corium, hypoplasia of the subcutis with increased mast cells and histiocytes), rosary-like tail, clubbed digits and deformed nails, squamous transformation of the palpebral epithelium and moderate congestion in certain viscera. Chemically, there was increased body content of water, ash and total nitrogen, diminution in fat and pronounced reduction in zinc.

Oral administration of inorganic zinc prevented these disorders, while vitamin B_1, certain vitamin B_2 compounds, liver pap and yeast had a mitigating effect. Therefore it was concluded that colostrum has a much higher content of zinc than later milk, and that deprivation of colostrum leads to manifestations of zinc deficiency in suckling mice.

ACKNOWLEDGMENTS

The author wishes to thank Dr. M. Araki for carrying out the zinc analyses, Dr. K. Nomura and Master S. Kato for their assistance concerning the chemical techniques, and Dr. Karl E. Mason for his kind advice and aid in the preparation of the manuscript.

LITERATURE CITED

BERTRAND, G., AND R. C. BHATTACHERJEE 1935 Recherches sur l'action combinee du zinc et des vitamines dans l'alimentation des animaux. Bull. soc. sci. hyg. aliment., *23:* 369–376.

BUNGER, H., AND BLOCKER 1930 Cited by W. Wohlbier, in Handbuch der Ernahrung und des Stoffwechsels der landwirtschaftlichen Nutztiere, von E. Mangold, 1932, *4:* 702.

DANIELS, A. L., AND M. K. HUTTON 1935 Mineral deficiencies of milk as shown by growth and fertility of white rats. J. Biol. Chem., *63:* 143–156.

DAY, H. G. 1942 The effects of zinc deficiency in the mouse. Fed. Proc., *1:* 188–189.

DAY, H. G., AND E. V. MCCOLLUM 1940 Effects of acute dietary zinc deficiency in the rat. Proc. Soc. Exp. Biol. Med., *45:* 282–284.

ENZMANN, E. V. 1933 Milk-production curve of albino mice. Anat. Rec., *56:* 345-358.

FOLLIS, R. H. 1948 The Pathology of Nutritional Disease. Charles C Thomas, Springfield, Ill.

FOLLIS, R. H., H. G. DAY AND E. V. MCCOLLUM 1941 Histological studies of the tissues of rats fed a diet extremely low in zinc. J. Nutrition, *22:* 223–237.

IMADA, S. 1940 Experimental studies on the effect of several heavy metals on the action of vitamin B_1. Part 3. The effect of zinc (jap.). Jikken-shokakibyogaku, *15:* 157–164.

JONES, J. H., C. FOSTER, F. DORFMAN AND G. L. HUNTER 1945 Effects on the albino mouse of feeding diets very deficient in each of several vitamin B factors (thiamine, riboflavin, pyridoxine and pantothenic acid). J. Nutrition, *29:* 127–136.

LIPPINCOTT, S. W., AND H. P. MORRIS 1941 Morphologic changes associated with pantothenic acid deficiency in the mouse. J. Natl. Cancer Inst., *2:* 39–46.

———— 1942 Pathologic changes associated with riboflavin deficiency in the mouse. Ibid., *2:* 601–610.

LUNDVALL, H. 1927 Farbung des Skelettes in durchsichtigen Weichteilen. Anat. Anz., *62:* 353–373.

NIELSEN, E., AND A. BLACK 1944 Biotin and folic acid deficiencies in the mouse. J. Nutrition, *28:* 203–207.

ORENT-KEILES, E., A. ROBINSON AND E. V. MCCOLLUM 1937 The effects of sodium deprivation on the animal organism. Am. J. Physiol., *119:* 651–661.

ORTEN, J., AND A. SMITH 1936 Goat's milk anemia. Yale J. Biol. Med., *8:* 637–642.

PARKES, A. S. 1929 Note on the growth of young mice suckled by rats. Ann. Appl. Biol., *16:* 171–173.

ROMINGER, E., AND C. BOMSKOV 1935 Untersuchungen an hyperchromen anamien bei experimentell erzeugten sprueartigen erkrankungen. Klin. Wschr., *14:* 148–150.

SMITH, T., AND R. B. LITTLE 1922 The significance of colostrum to the newborn calf. J. Exp. Med., *36:* 181–198.

SPRAY, C. M. 1950 A study of some aspects of reproduction by means of chemical analysis. Brit. J. Nutrition, *4:* 354–360.

SPRAY, C. M., AND E. M. WIDDOWSON 1950 The effect of growth and development on the composition of mammals. Ibid., *4:* 332–353.

STIRN, F. E., C. A. ELVEHJEM AND E. B. HART 1935 The indispensability of zinc in the nutrition of the rat. J. Biol. Chem., *109:* 347–359.

SULLIVAN, M., AND V. J. EVANS 1944 Nutritional dermatoses in the rat. IX. Evaluation of the interrelationship of magnesium deficiency and deficiencies of the vitamin B complex J. Nutrition, *27:* 123–139.

TAKUMA, T. 1941 On the infant feeding (jap.). Jikazasshi, *47:* 1071–1096.

TODD, W. R., C. A. ELVEHJEM AND E. B. HART 1934 Zinc in the nutrition of the rat. Am. J. Physiol., *107:* 146–156.

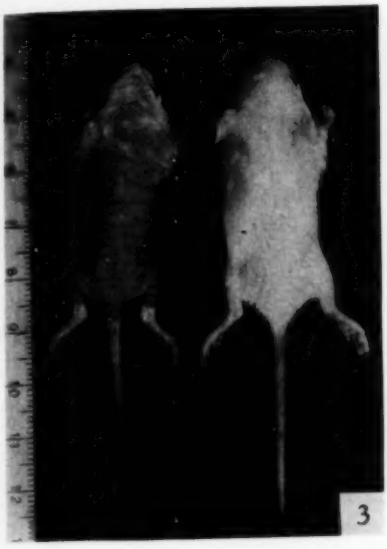
PLATE 1

EXPLANATION OF FIGURES

3 The mouse on the left is a foster suckling at the 15th day of age with denuded, glossy and wrinkled skin on the back and neighboring areas, which was reared from birth by a foster mother 15 days post-parturient. The mouse on the right is a 15-day normal suckling of the same litter reared by its own mother.

4 Foster suckling at the 18th day of age, showing alopecia on the posterior dorsum, a rosary-like tail and clubbed fingers.

5 Profiles of the digits of a normal suckling (on the left) and a foster suckling (on right) of the same litter at the 14th day of age. Note the stronger plantar curvature of the nail in the latter. × 20.


3

4

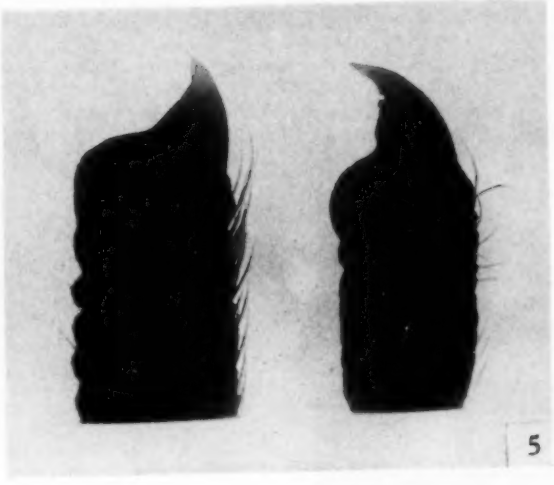

5

PLATE 2

EXPLANATION OF FIGURES

6 Dorsal skin of a normal suckling at the 11th day of age. Methyl green-pyronin. × 100.

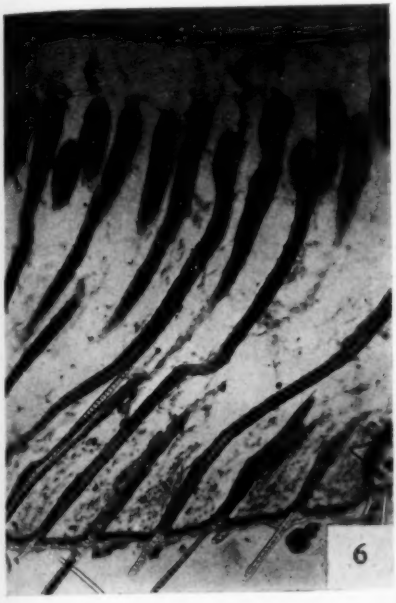
7 Dorsal skin of a foster suckling of the same litter at the 11th day of age. Thickened epidermis, desquamated corneum, sparse and atrophic hair follicles, thinner subcutaneous layer and increased mast cells. Methyl green-pyronin. × 100.

8 Transverse section of the tail of a normal suckling at the 14th day of age. Hematoxylin and eosin. × 75.

9 Transverse section of a rosary-like tail of a foster suckling at the 14th day of age, showing remarkable thickening of the epidermis with degenerated nuclei, sparse hairs with no medullary substance and congestion of the capillaries throughout the skin and subcutaneous layer. Hematoxylin and eosin. × 45.

10 Palpebral conjunctiva of a normal suckling at the 14th day of age with stratified cuboidal epithelium. Hematoxylin and eosin. × 450.

11 Palpebral conjunctiva of a foster-suckling at the 14th day of age with stratified squamous epithelium. Heamtoxylin and eosin. × 450.


6

7

9

8

10

11

DERMATOSIS IN WEANLING RATS FED LACTOSE DIETS

II. HISTOLOGICAL STUDIES [1]

H. NIÑO-HERRERA,[2] M. SCHREIBER, R. A. COLLINS[3] AND C. A. ELVEHJEM

Department of Biochemistry, University of Wisconsin, Madison

SEVEN FIGURES

(Received for publication July 26, 1952)

INTRODUCTION

The scaly paw condition reported by Geyer et al. ('43) and Boutwell et al. ('44, '45), which was observed when weanling albino rats were fed corn oil-lactose diets, has recently been observed to occur in animals fed diets of mineralized cow's milk and goat's milk (Collins, '50; Collins et al., '51). The maximum degree of scaly dermatosis appeared when weanling rats were fed the lactose diets for 10 to 12 days; after this time the animals spontaneously recovered from the dermatosis in an additional two to three weeks. Further studies by Schreiber et al. ('52) have shown that the severity of the scaliness increases with low relative humidity, and that scales do not appear when lactose-fed rats are maintained in an environment with a relative humidity of more than 50%.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by funds granted by the National Dairy Council, Chicago, on behalf of the American Dairy Association; and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[2] Rockefeller Foundation fellow, from the National University of Colombia, Bogota.

[3] Present address: International Minerals and Chemical Corporation, Central Research Laboratory, Skokie, Illinois.

This report is concerned with the histological changes which occur in the dorsal skin of the hind paws of weanling rats fed a cow's milk diet, when maintained at low relative humidity and at high relative humidity.

EXPERIMENTAL

Two groups of male weanling rats, weighing 35 to 40 gm, of the Sprague-Dawley strain, were placed in raised screen-bottom group cages and were maintained at constant humidity conditions: group 1 at 12 to 24% relative humidity, and group 2 at 65 to 75% relative humidity. A constant temperature of 26°C. was maintained throughout the experimental period. The controlled-humidity chambers and their operation are described by Schreiber et al. ('52).

Both groups of animals were fed mineralized (Fe, Cu and Mn) raw cow's milk. The cow's milk was obtained from the University of Wisconsin Creamery and was fed ad libitum each morning and evening. One animal from each group was sacrificed daily for three weeks. Post-mortem examinations were performed and skin samples from the dorsal side of the hind paws were taken for histological examination. The skin samples were immediately fixed and paraffin sections were prepared. From each sample a continuous zone of about 700 μ was studied by means of horizontal, vertical and diagonal sections 6 μ thick. The samples were fixed with Bouin's fixer, and the slides were stained using the following techniques: hematoxylin-eosin, Cajal's polychromatic, and these two combined.

RESULTS

In accord with previous experiments, the gross skin changes occured in a normal sequence in the animals of group 1 which were exposed to the low humidity environment. These rats showed dryness of the skin of the fore and hind paws by the second day and cracking of the skin of the paws by the third to the 5th day. Manifestations of scaly dermatosis were apparent on the paws by the 7th to 8th day and reached a maxi-

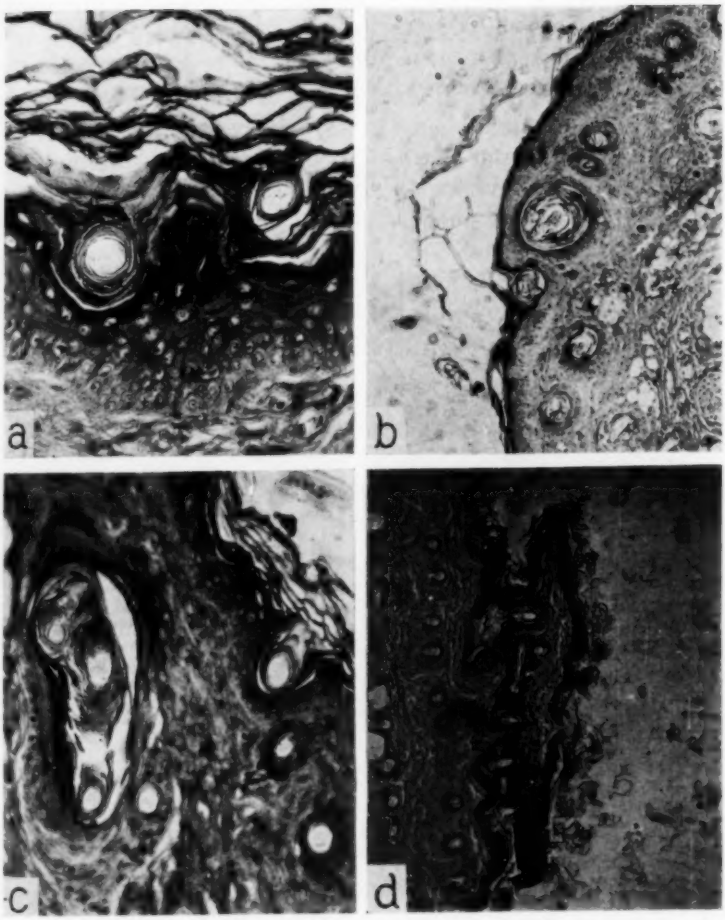


Fig. 1 a Hyperkeratosis, hyperchromatosis and cell vacuolization. Sample from high relative humidity, 6th day.

Fig. 1 b Hair follicle hyperkeratinization and degenerative hypertrophy. Sample from low relative humidity, 6th day.

Fig. 1 c Structural continuity of affected hair follicles. Sample from low relative humidity, 7th day.

Fig. 1 d Scale structure. Sample from low relative humidity, 10th day.

mum severity by the 10th to 12th day. Complete spontaneous recovery from the scaly dermatosis occurred in an additional two to three weeks.

The rats of group 2 which were maintained at the high humidity did not show any gross evidence of scaliness during the experimental period. In both groups a slight diarrhea was present during the third to 6th day. Post-mortem examination showed mild intestinal congestion from the third

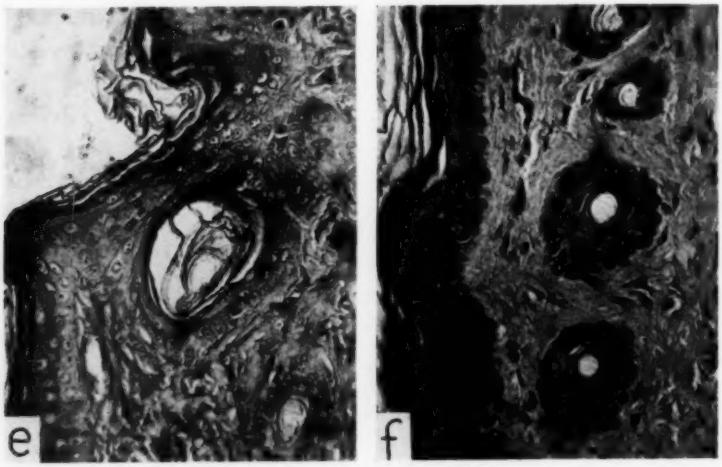


Fig. 2 e Similar to 1 b, but from high relative humidity.
Fig. 2 f Normal skin, stock diet.

to the 7th day. The size of the cecum was observed to increase progressively with a corresponding change in the cecal contents to a more fluid consistency and a change of the color of the contents from blue-grey to yellow-brown.

The histological alterations in the dorsal side of the hind paws were found to be similar at both high and low humidity, except that the changes in the rats of group 2 (high humidity) appeared from one to two days later than those in the animals of group 1 (low humidity). The first evidence of a change in the skin structure was observed after the

animals had received the cow's milk diet for a period of three days. During the third to 6th day hyperkeratinization of the hair follicles, protoplasmic vacuolization, and a tendency toward hyperchromatosis were observed in the cells

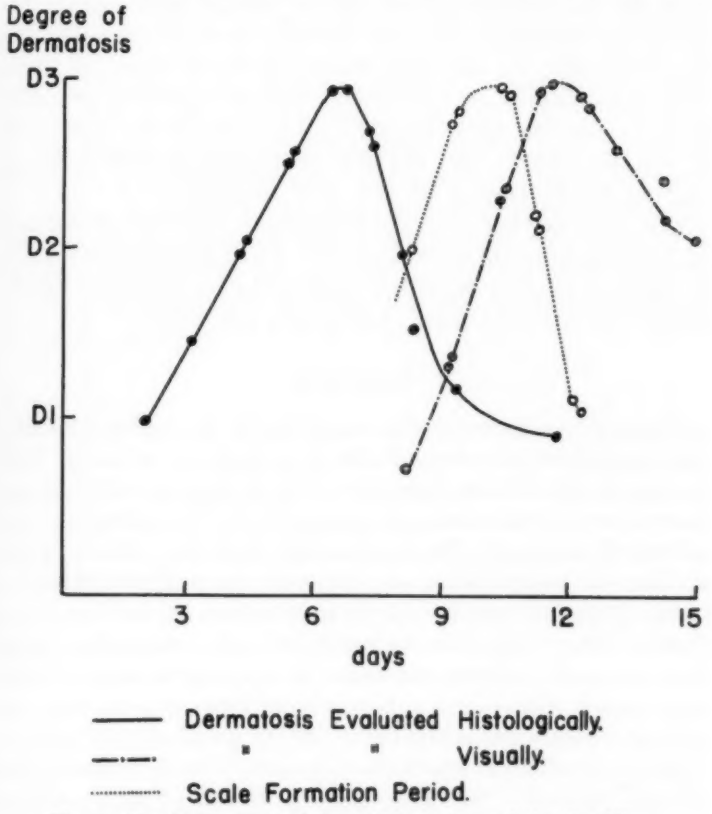


Fig. 3 The development of dermatosis at low relative humidity.

of the stratum granulosum (fig. 1 a). Modifications in the structure of the hair follicles were, however, the most significant changes (fig. 1 b, c and d and fig. 2 e). The cells of the capillaries and the other skin cells were apparently

not altered during the course of this dermatosis. Figure 2 f shows a section of normal skin.

The extent of the cellular alterations is graphically illustrated in figure 3, using the arbitrary designations of D1 for mild, D2 for moderate, and D3 for severe dermatosis. The maximum change in the hair follicles was observed by the 6th to 8th day. At this time thickening of the cornified layer, with some degree of exfoliation, and an apparent plugging of the sebaceous ducts could be seen. Also at the same time it was possible to see the differentiation of new cells, including the formation of new hair follicles.

The time lag between the histological and visual changes of dermatosis is shown in figure 3. The appearance of scales at the low relative humidity (group 1) occurred simultaneously with the formation of new hair follicles.

DISCUSSION

Since the results of this experiment have demonstrated that hyperkeratinization of the hair follicles occurred independently of relative humidity, dehydration of the skin surface from without does not appear to be the primary cause of this dermatosis. The biochemical basis for this dermatosis has not been elucidated; however, the work of Schreiber et al. ('52) has implicated dietary lactose as one important factor. The formation of enlarged ceca containing acidic fluid material, and the presence of varying degrees of diarrhea, which occurred in rats receiving lactose diets, may contribute directly or indirectly to the origin of the dermatosis.

The formation of the scales appears to be dependent upon several factors. The arrangement of the hyperkeratinized hair follicles, as shown in figure 1 b, c and d may aid the scale formation. The temporary dysfunction of the sebaceous glands, leaving the skin without its normal secretion products, may also favor surface dehydration when the relative humidity is low. The dry cornified layer ruptures, thus initiating the scale formation.

This dermatosis may be distinguished from the other known nutritional dermatoses by the rapid appearance of the histological changes and by the spontaneous recovery. Dermatosis produced by a fat-free diet also showed increased keratinization, but the time of appearance was considerably later (Williamson, '41). In the case of the dermatosis resulting from a riboflavin deficiency, a different localization of the affected tissues was observed (Sullivan and Nicholls, '41). Dermatosis produced by a pyridoxine deficiency occurred at a later time and with a different picture of the altered tissues (Sullivan and Nicholls, '40). The scaly dermatosis observed in vitamin A deficiency shows some points of similarity with the dermatosis reported here, but the intensity of the tissue changes produced by the vitamin A deficiency is greater and the time of appearance and the duration of the scaliness are different (Sullivan and Evans, '45).

SUMMARY

Histological studies performed upon the skin from the dorsal side of the hind paws of weanling rats fed a mineralized cow's milk diet revealed similar changes at both high and low relative humidity; however, scaliness did not occur at high relative humidity. The principal change in the structure of the skin was observed to be a hyperkeratinization of the hair follicles. The spontaneous recovery from scaliness which occurred at low relative humidity was preceded by a return of the skin to its normal structure.

LITERATURE CITED

BOUTWELL, R. K., R. P. GEYER, C. A. ELVEHJEM AND E. B. HART 1944 Effect of flavor on nutritive value of fats. Proc. Soc. Exp. Biol. Med., *55:* 153–155.

———— 1945 Studies on the interrelation of fats, carbohydrates and B-vitamins in rat nutrition. Arch. Biochem., *7:* 143–157.

COLLINS, R. A. 1950 The significance of folic acid and vitamin B_{12} in milk diets. Master's Thesis, University of Wisconsin.

COLLINS, R. A., M. SCHREIBER AND C. A. ELVEHJEM 1951 Occurrence of dermatitis in rats fed diets containing lactose as the sole carbohydrate. Fed. Proc., *10:* 379.

GEYER, R. P., R. K. BOUTWELL, C. A. ELVEHJEM AND E. B. HART 1943 Rations for the study of the relative nutritive value of fats and oils. Science, *98:* 499.

SCHREIBER, M., R. A. COLLINS, H. NIÑO-HERRERA AND C. A. ELVEHJEM 1952 Dermatosis in weanling rats fed lactose diets. I. The influence of relative humidity and diet. J. Nutrition, *48:* 125.

SULLIVAN, M., AND V. J. EVANS 1945 Nutritional dermatoses in the rat. XI. Vitamin A deficiency superimposed on vitamin B complex deficiency. Arch. Dermat. Syph., *51:* 17–25.

SULLIVAN, M., AND J. NICHOLLS 1940 Nutritional dermatoses in the rat. I. Vitamin B_6 deficiency. J. Investigative Dermatol., *3:* 317–335.

———— 1941 Nutritional dermatoses in the rat. IV. Riboflavin deficiency. Ibid., *4:* 181–191.

WILLIAMSON, R. 1941 A note on the epidermis of the rat on a fat free diet. Biochem. J., *35:* 1003–1005.

RAT GROWTH ASSAY FOR VITAMIN B_{12}. CORRESPONDENCE WITH COLLABORATIVE MICROBIOLOGICAL ASSAY RESULTS ON U.S.P. LIVER EXTRACTS

DOUGLAS V. FROST, HOWARD H. FRICKE
AND HENRY C. SPRUTH
Abbott Laboratories, North Chicago, Illinois

TWO FIGURES

(Received for publication August 11, 1952)

A growth assay for vitamin B_{12} in depleted rats on a casein-dextrin diet with iodinated casein has been reported from this laboratory (Frost, Spruth and Fricke, '49). Except for the diet used, the assay is similar to that described by Register, Ruegamer and Elvehjem ('49). Zucker and Zucker ('50) have recently reviewed methods of vitamin B_{12} assay in animals. Following our original report, various improvements have been made in the basal diet during two years of experimentation with the method.

An outstanding opportunity to correlate the rat assay method with the microbiological assay came when Dr. Lloyd Miller, chairman of the U.S.P. Revision Committee, made available to us samples of liver extract used in the recent collaborative assay in which 26 laboratories participated. The microbiological assay procedure used by the collaborating laboratories is described in the Pharmacopoeia of the U. S., XIV, third supplement ('51). Essentials of the assay, together with a description of the assay samples (Miller, '52) and a statistical analysis of the data of the collaborative study (Brownlee, '52), are available. We are indebted to Dr. Brownlee and to Dr. Lloyd Miller for an opportunity to study this

analysis, prior to publication, in connection with the significance of the work herein described.

Preliminary rat assays on the U.S.P. liver samples were made, where we used microbiological assay values determined by Eleanor Willerton and H. W. Cromwell by the method of Skeggs et al. ('50). This method has appeared to give reliable results in routine use in this laboratory. The microbiological assay values obtained, together with the average U.S.P. values from the 26 laboratories, are shown in table 1.

TABLE 1

Average microbiological assay results of U.S.P. liver extract samples

SAMPLE [1]	AVERAGE OF 26 LABORATORIES [2]	ABBOTT ROUTINE ASSAY VALUES
	μg per ml	*μg per ml*
Liver extract 1	14.86	16.0
3	11.05	11.8
2	0.838	0.9
5	0.637	0.75
6	11.21	12.5
4	8.74	8.5

[1] Liver extract samples 3, 5, and 4 are 80% dilutions of samples 1, 2, and 6, respectively.

[2] These values are geometric means of the 4 assays conducted by each laboratory.

The preliminary rat assays were not entirely satisfactory as to the experimental design; however, the suggestion was clear that rat growth response was in accord with microbiological vitamin B_{12} activity. When the U.S.P. collaborative results became available and the sample dilutions were known, we set up a more complete assay of two representative samples, as described below.

EXPERIMENTAL

Rats used in this study were from the laboratory colony. These animals, originally Wistar, have been inbred over a period of 20 years on the following stock diet: wheat meal (entire kernel) 33.0; yellow corn meal (entire kernel) 34.0; whole milk powder 21.0; old-process linseed oil meal 7.0;

alfalfa leaf flour (green color) 2.0; calcium carbonate 0.5; sodium chloride 0.5; liver (vacuum-dried) 1.0; salts 0.5; and brewers' yeast 0.25%. Fresh lettuce is fed separately once a week.

Weanling rats of mixed sex from the Abbott colony were placed on the basal diet for 11 days for depletion before assay. The basal mixture of the assay diet consisted of the following in per cent: casein [1] 18, dextrin 71, salt mixture 4, cellu flour 2, agar 1.5, hydrogenated vegetable oil [2] 2, cod liver oil 1, sulfaguanidine 0.5, iodinated casein [3] 0.05, choline chloride 0.1. This basal mixture was supplemented with the following (in milligrams per 100 gm of basal mixture): riboflavin 3, thiamine hydrochloride 3, niacin 3, pyridoxine hydrochloride 5, folic acid 0.5, calcium DL-pantothenate 5.0, *p*-aminobenzoic acid 5.0, menadione 0.5, inositol 20, D-biotin 0.01, $CuSO_4 \cdot 5H_2O$ 4, and $ZnSO_4 \cdot 7H_2O$ 4.

Previous work (Frost et al., '49) showed that vitamin B_{12} is equally active whether given orally or by injection under the assay conditions employed. For convenience, therefore, assays are conducted by simply adding test samples to the basal diet for comparison with varying levels of crystalline vitamin B_{12} likewise added to the diet. Suitable aliquots of the test materials — i.e., 2 to 10 ml — are dried on 50 to 200 gm of powdered dextrin. The dextrin is then thoroughly blended into weighed amounts of the basal diet. The rats are fed ad libitum and weighed weekly.

Activity of vitamins B_{12} and B_{12b}

There is some evidence that hydroxycobalamin rather than cyanocobalamin is the dominant form of vitamin B_{12} in liver extract. Experiments in this laboratory demonstrate, as shown in figure 1, that there is no difference in the rat activity between these crystalline forms of the vitamin.

[1] Vitamin-test, General Biochemicals, Inc.

[2] Primex.

[3] Protamone, courtesy of George Kohler, Cerophyl Laboratories, Kansas City.

Assay of U.S.P. liver extract samples

For the detailed assay of two of the collaborative U.S.P. samples, 140 weanling rats of mixed sex, weight range 37 to 45 gm, were depleted 11 days on the basal diet. Following depletion, groups of 14 rats were formed of equal average weight. The diet of one group was unsupplemented. Similar groups were placed on diets containing graded amounts of vitamin B_{12}, or liver extract samples at levels calculated to

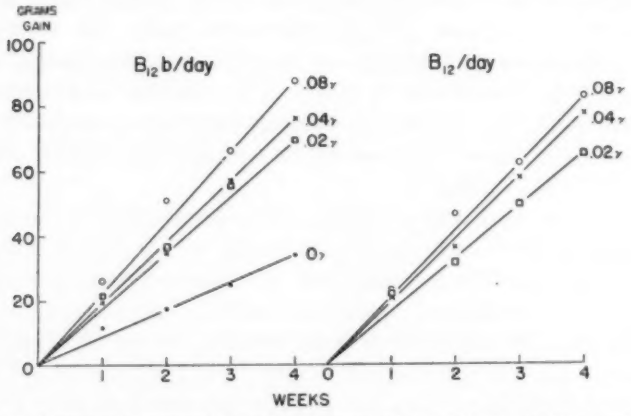


Fig. 1 Four-week average growth responses of vitamin B_{12}-depleted rats to injections of critical graded allowance of vitamin B_{12} or B_{12b}. Injections of solutions of the crystalline vitamins were made thrice weekly to provide the per diem amounts shown. Five male and 5 female rats were used in each group.

provide identical amounts of vitamin B_{12} activity. The results of the assay are shown in figure 2. The correspondence between the growth increments elicited by the liver extract samples and by crystalline vitamin B_{12} in this experiment is remarkably close. The standard errors calculated at the end of the third week of assay ranged from 2.4 to 3.3 gm. Of 140 rats started, 133 survived the experiment. Three of the fatalities were in the control group.

Two other tests of the correspondence between rat and microbiological assays of the collaborative U.S.P. liver ex-

tract samples have furthered the conclusion that both tests measure vitamin B_{12} activity only. The vitamin B_{12}-sparing action of antibiotics was tested in conjunction with one of these runs. Table 2 shows the average two-week weight gain of both the male and female rats for comparison. In general the males gained more than the females, but this was not

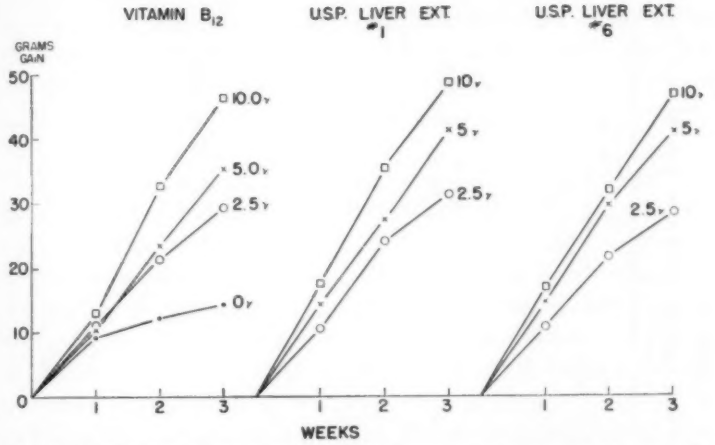


Fig. 2 Comparison of the average weight responses of groups of 14 rats each to supplementation of the diet with crystalline vitamin B_{12} or its microbiological assay equivalent in terms of two different collaborative U.S.P. liver extract samples. The figures to the right of each set of curves indicate the micrograms of vitamin B_{12} added per kilogram of basal diet.

uniformly true. Only female rats were used in the test involving procaine penicillin. Subsequent tests have shown that procaine penicillin exerts a remarkable sparing effect for vitamin B_{12} in the rat, regardless of sex.

Assay of liver extract samples treated to destroy vitamin B_{12}

It was of interest to use the rat assay as a check on methods of destroying vitamin B_{12} in liver extracts. The suggestion had been made that vitamin B_{12} can be destroyed by treatment

TABLE 2

Further assay of U.S.P. liver extracts with male and female rats. Sparing effect of antibiotics

SUPPLEMENT AND NO. OF RATS PER GROUP	AVERAGE 2-WEEK WEIGHT GAIN AT THREE LEVELS OF VITAMIN B_{12} PER KG DIET		
	0 μg	2.5 μg	5.0 μg
Negative control			
Males (5)[1]	15.6 (4)		
Females (5)	19.7 (4) ave. 17.7		
U.S.P. B_{12} reference standard			
Males (5)		25.4 (5)	37.7 (4)
Females (5)		18.0 (4) ave. 22.1	24.8 (4) ave. 31.1
U.S.P. liver extract 1			
Males (5)		17.8 (5)	41.9 (5)
Females (5)		29.6 (3) ave. 22.2	36.0 (5) ave. 38.4
U.S.P. liver extract 2			
Males (5)		19.7 (4)	33.2 (4)
Females (5)		21.4 (5) ave. 20.6	22.8 (5) ave. 27.4
U.S.P. liver extract 6			
Males (5)		25.8 (5)	32.8 (5)
Females (5)		21.2 (5) ave. 23.5	29.4 (5) ave. 31.1
Procaine pen., 100 mg per kg			
Females (6)	34.5 (6)		
Aureomycin HCl., 100 mg per kg			
Males (5)	32.3 (3)		
Females (5)	32.0 (5) ave. 32.2		

[1] Figures in parentheses represent numbers of rats.

with alkali, and that the residual activity in liver extracts may be ascribed to the presence of desoxyribosides. To test this idea, 10 U.S.P. units of liver extracts containing about 10 μg vitamin B_{12} per milliliter were made to pH 11 to 12 with sodium hydroxide, and heated one hour at 100°C. A similar sample was treated twice with 10% (w/v) additions of sodium hydrosulfite at 5°C., as an alternative way of destroying vitamin B_{12}. Microbiological assay showed 2 to 5% residual B_{12} activity in the liver extract treated by both methods. The residues were assayed in rats at levels corresponding to the microbiological assay values, assuming all of the activity to be true vitamin B_{12}.

The rat growth response corresponded in each case to a level of about 0.5 μg B_{12} per milliliter of liver extract, the approximate level shown by microbiological assay. Cup plate assays conducted by Thomas J. Oliver showed zones of growth typical for vitamin B_{12}. The data suggest that destruction of vitamin B_{12} is incomplete under these conditions. It is of significance to note that thymidine, a desoxyriboside normally present in liver extract, did not elicit a growth response in two separate assays at a level of 50 mg per kilogram of diet.

Effect of nicotinamide in diet

Excess nicotinamide in the diet places an added strain on the ability to provide methyl groups for the formation of its excretion products. One milligram of nicotinamide per gram of body weight per day was found to produce fatty livers in young rats on a purified diet (Handler and Dann, '42; Frost, '47). When 1% nicotinamide was added to the diet under the present assay conditions, the growth of both control and vitamin B_{12}-deficient animals was markedly depressed. Addition of 0.5% nicotinamide to the diet also decreased growth in each instance. However, the spread in weight gain between the deficient and supplemented animals was greater at the 0.5% than at the 1% nicotinamide level. The results using

the 0.5% nicotinamide level are seen in table 3. Piebald rats[4] were used in a similar experiment with good results.

DISCUSSION

The present diet represents certain improvements over that previously used (Frost et al., '49). The fat level has been decreased from 6 to 3% in line with the reported sparing effect of fat for the vitamin B_{12} requirement (Bosshardt et al., '50). The Jones and Foster salt mixture ('42) was substituted for salt mixture no. 1 (Osborne and Mendel) of

TABLE 3

Effect of excess nicotinamide on response to vitamin B_{12}

SUPPLEMENT TO DIET (10 rats per group)	STARTING WEIGHT, AVE.	TOTAL WEIGHT GAIN			NO. OF RATS SURV.	STD. ERROR, 3RD WEEK
		1st week ave.	2nd week ave.	3rd week ave.		
	gm	*gm*	*gm*	*gm*		*gm* ±
None	54.7	7.5	11.4	19.3	7	4.4
Vitamin B_{12}, 1.5 μg/kg	56.3	8.5	17.8	27.6	7	2.2
Vitamin B_{12}, 3 μg/kg	56.2	10.2	22.5	40.2	9	3.7
Vitamin B_{12}, 6 μg/kg	56.2	11.9	30.7	48.7	10	3.0
Vitamin B_{12}, 12 μg/kg	56.1	17.4	40.3	59.2	10	3.0
Nicotinamide, 0.5%	56.1	— 0.6	5.4	10.4	8	4.1
Nicotinamide, 0.5% plus vitamin B_{12}, 12 μg/kg	56.1	9.3	25.2	46.0	8	4.7

U.S.P. XIII. This diet provides improved levels of calcium and phosphorus plus adequate amounts of copper, cobalt and zinc. The level of folic acid has been increased, and biotin has been added. Elimination of either sulfaguanidine or *p*-aminobenzoic acid in single runs did not appear to alter the course of the assay. Mortality is still a somewhat unpredictable factor in the assay, making it desirable to use not less than 10 rats per group.

Various crude and purified materials were ineffective in stimulating growth above that obtained on the basal diet.

[4] Obtained from Rockland Farms.

These include: choline, cystine, methionine, folic acid, *p*-aminobenzoic acid, primary grown and debitterized brewers' yeasts, thymidine, and a concentrate of vitamin B_{13}.[5]

The addition of 3,4-dimethyl benzimidazole, plus $CoCl_2 \cdot 6H_2O$, each at 10 mg per kilogram of diet, gave a small but significant growth stimulation in our assay. Emerson et al. ('51) and Cooperman and Tabenkin ('51) have reported the fractional activity of many possible derivatives of vitamin B_{12} as substitutes for vitamin B_{12} in the rat assay. Hartman et al. ('49) had previously reported the similar effect of high levels of riboflavin. It does not seem likely, however, that any of these substances would interfere in the present assay, except under extraordinary conditions.

Tappan et al. ('50) and Cravioto-Munoz et al. ('51) first described the sparing action of aureomycin for vitamin B_{12} in the rat. Oleson et al. ('50) reported a similar effect in chicks. Our experiments, in which a marked sparing action of penicillin and aureomycin for vitamin B_{12} was observed, will be published in detail elsewhere. Thus it is clear that the presence of antibiotics may complicate the rat assay for vitamin B_{12}.

When the level of iodinated casein in the basal diet was increased from 0.05 to 0.3%, the growth-stimulating effect of vitamin B_{12} was diminished or disappeared altogether. Under these rigorous conditions, liver residue at levels of 5 to 10% of the diet gave increased growth and decreased mortality, in accord with the findings reported by Ershoff ('50). On the other hand, liver residue did not give greater growth stimulation under the conditions of our vitamin B_{12} assay than was accounted for by its content of vitamin B_{12} as determined by microbiological assay.

The results of these studies appear to support our previous statement (Frost et al., '49) indicating that where both male and female rats are used, there should be equal distribution as to sex. Watts et al. ('51) studied the effect of sex on

[5] Sample of vitamin B_{12} concentrate from distillers' solubles obtained from Dr. J. Van Lanen of Hiram Walker and Co., Peoria, Ill., for vitamin B_{12} assay.

response to vitamin B_{12} in the presence of 0.25% iodinated casein in the diet. They suggested that we had not paid adequate attention to sex differences in our first paper. This criticism does not appear justified in view of our statement on this very point. Furthermore, the high level of iodinated casein used by Watts et al. ('51) exerts markedly different physiological effects than the relatively low level used in our assay for vitamin B_{12}. Significantly, Ershoff ('50) observed no growth response to vitamin B_{12} in the presence of high levels of thyroxin. In our experience with the vitamin B_{12} assay, equal sex distribution appears essential. The use of only one sex is important where the number of rats per group is less than 10.

Use of nicotinamide in the assay diet may accentuate the need for vitamin B_{12}, but further work is needed to establish its usefulness. Dinning et al. ('50) studied the effects of 1% pyridine in the diet on the growth of rats in the presence and absence of vitamin B_{12} or folacin or both. As in the present experiments, addition of vitamin B_{12} improved growth under this condition of physiologic stress.

Rats from different sources have responded differently in the assay in our laboratory. Rats from our own colony, inbred on a uniform stock diet for many years, have given reproducible assays without exception over a period of three years. When rats were purchased from three different sources, only one of the groups gave a satisfactory assay. It appears likely that maternal diet is most important in the preparation of weanling rats for this assay. Other variables, such as strain and the diet of the rats during transit, may play a part.

Doctor Elizabeth Miller of the McArdle Institute, University of Wisconsin, reported failure to reproduce our assay results with purchased rats. Using the basal diet sent from our laboratory, Dr. Miller reported that there was still no difference in growth rate for the B_{12}-supplemented and unsupplemented animals. Using both diet and rats shipped from our laboratory, Dr. Miller reported satisfactory B_{12} depletion. The data, which Dr. Miller offered us for publication, are as

follows: 20 weanling rats were depleted on the basal diet for 14 days. One rat died on the 13th day. The average gain for 14 days was 33 gm, range 22 to 47 gm. Eight rats were placed on diet with 20 μg vitamin B_{12} per kilogram and 11 rats were kept on the deficient diet. After 18 days, 4 surviving control rats averaged 22 gm, range 4 to 35 gm, whereas 6 supplemented rats averaged 66 gm, range 60 to 74 gm. Doctor Miller's experiments revealed a manifest difference in the metabolism of the two groups.

Girdwood and Carmichael ('50) correlated the clinical response in pernicious anemia patients with the vitamin B_{12} content of 15 liver extracts, as determined by microbiological assay. They tentatively summarize as follows: "The results give no evidence to suggest that the hemopoietic responses cannot be attributed to the vitamin B_{12} content of the liver extracts." Hartman et al. ('49) concluded, in studies with rats wherein they compared crystalline vitamin B_{12} with liver extract, that the effective growth-promoting material in the liver extract was B_{12} or B_{12}-active material.

Pierce et al. ('50), and Fricke et al. ('50) have reported the equal activity of vitamins B_{12} and B_{12b} in animals and in man. Kaczka et al. ('50) have recognized the identity of vitamins B_{12a} and B_{12b} and have shown that various forms of the vitamin, other than cyanocobalamin, have equal activity. The product formed by interaction of vitamin B_{12} or B_{12b} with sulfite (Fricke et al., '50) has proved fully active microbiologically and in rats in this laboratory. On the other hand, a recent report by Pfiffner et al. ('51) points to at least two cobalt-containing pigments belonging to the vitamin B_{12} group which are active microbiologically but inactive in rats and chicks. These compounds, which the authors term "crystalline pseudovitamin B_{12}," were isolated as products of anaerobic fermentation produced by organisms from bovine rumen contents. It will be important to determine whether these materials may complicate the microbiological assay of liver extracts. Along this line, it is of interest that Tappan, Lewis, Register and Elvehjem ('50) have reported

good correspondence between rat and microbiological assay methods on a variety of fish and meat tissues.

SUMMARY

A method of rat growth assay for vitamin B_{12} using a highly purified diet with casein has been further studied and improved. Three liver extract samples, used in the 1950 U.S.P. collaborative study of the microbiological vitamin B_{12} assay, were assayed in rats at levels corresponding to the average values of the 26 participating laboratories. The growth responses for the three liver extracts at two and three levels in the diet were in line with the response to similar graded levels of crystalline vitamin B_{12}. These results support the authenticity of the U.S.P. microbiological assay for vitamin B_{12} as applied to injectable liver extracts. It should be borne in mind that antibiotics spare the requirement for vitamin B_{12} and offer a possible interference in the rat assay.

The method was used to test the specificity of the microbiological method as a measure of the completeness of destruction of vitamin B_{12} in liver extract by alkali.

Nicotinamide added to the diet depressed the growth of control and supplemented animals, but did not necessarily improve the sensitivity of the assay.

ACKNOWLEDGMENTS

We are grateful to Harry Sandy, Albin Junnila and Burton Main for technical aid in the conduct of the study.

LITERATURE CITED

BOSSHARDT, D. K., W. J. PAUL AND R. H. BARNES 1950 The influence of diet composition on vitamin B_{12} activity in mice. J. Nutrition, *40:* 595.

BROWNLEE, K. A. 1952 A statistical analysis of the 1950 collaborative study on the U.S.P. method for vitamin B_{12} assay. Drug Standards, *20:* 48.

COOPERMAN, J. M., AND B. TABENKIN 1951 Vitamin B_{12} activity of 5,6-dimethylbenzimidazole, 1,2-diamine-4,5-dimethylbenzene, and riboflavine for the rat. Fed. Proc., *19:* 175.

CRAVIOTO-MUNOZ, J., H. G. PONCHER AND H. A. WAISMAN 1951 Vitamin B_{12} sparing action of aureomycin in the rat. Proc. Soc. Exp. Biol. Med., *77:* 18.

DINNING, J. K., C. K. KEITH, J. T. PARSONS AND P. L. DAY 1950 The influence of pteroylglutamic acid and vitamin B_{12} on the metabolism of pyridine-fed rats. J. Nutrition, *42:* 81.

EMERSON, G., F. W. HOLLY, C. H. SHUNK, N. G. BRINK AND K. FOLKERS 1951 Vitamin B_{12}. XII. Vitamin B_{12}-like activity of alpha and beta-ribazole. J. Am. Chem. Soc., *73:* 1069.

ERSHOFF, B. H. 1950 Effects of vitamin B_{12} and liver residue on growth of hyperthyroid male rats. Proc. Soc. Exp. Biol. Med., *75:* 459.

FRICKE, H. H., B. LANIUS, A. F. DEROSE, M. LAPIDUS AND D. V. FROST 1950 Comparison of vitamin B_{12} and vitamin B_{12a}. Fed. Proc., *9:* 173.

FROST, D. V. 1947 The effect of nicotinamide on the solubility of riboflavine in water. J. Am. Chem. Soc., *69:* 1064.

FROST, D. V., H. C. SPRUTH AND H. H. FRICKE 1949 Rat growth assay for vitamin B_{12}. Proc. Soc. Exp. Biol. Med., *72:* 102.

GIRDWOOD, R. H., AND K. M. CARMICHAEL 1950 The content of hematopoietic factors in liver extracts, relationship to clinical response. Brit. Med. J., *2:* 1357.

HANDLER, P., AND W. J. DANN 1942 The inhibition of rat growth by nicotinamide. J. Biol. Chem., *146:* 357.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 The role and sources of vitamin B_{12}. J. Am. Diet. Assn., *25:* 929.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either high or low in phosphorus. J. Nutrition, *24:* 245.

KACZKA, E. A., D. E. WOLF, F. A. KUEHL AND K. FOLKERS 1950 Vitamin B_{12}: reactions of cyano-cobalamin and related compounds. Science, *112:* 354.

MILLER, L. C. 1952 The 1950 U.S.P. collaborative study of the vitamin B_{12} assay. Drug Standards, *20:* 41.

OLESON, J. J., B. L. HUTCHINGS AND A. R. WHITEHILL 1950 The effect of feeding aureomycin on the vitamin B_{12} requirement of the chick. Arch. Biochem., *29:* 334.

PFIFFNER, J. J., D. G. CALKINS, R. C. PETERSON, O. D. BIRD, V. MCGLOHON AND R. W. STIPEK 1951 Crystalline pseudovitamin B_{12}. Abstr., Am. Chem. Soc. meeting, New York, Sept., p. 22c.

PHARMACOPOEIA OF THE UNITED STATES 1951 14th Revision, 3rd Supplement, p. 15.

PIERCE, J. V., A. D. PAGE, E. L. R. STOKSTAD AND T. J. JUKES 1950 Studies of some characteristics of vitamin B_{12b}. J. Am. Chem. Soc., *72:* 2615.

REGISTER, U. D., W. R. RUEGAMER AND C. A. ELVEHJEM 1949 An improved assay for growth in liver extracts. J. Biol. Chem., *177:* 129.

SKEGGS, H. R., H. M. NAPPLE, K. A. VALENTIK, J. W. HUBB AND L. D. WRIGHT 1950 Observations on the use of *Leichmanii* 4797 in the microbiological assay of vitamin B_{12}. Ibid., *184:* 217.

TAPPAN, D. V., U. J. LEWIS, U. D. REGISTER AND C. A. ELVEHJEM 1950 Further studies on the rat growth assay for vitamin B_{12} activity. Arch. Biochem., *29:* 408.

WATTS, A. B., O. B. ROSS, C. K. WHITEHAIR AND R. W. MACVICAR 1951 Response of castrated male and female hyperthyroid rats to vitamin B_{12}. Proc. Soc. Exp. Biol. Med., *77:* 624.

ZUCKER, T. F., AND L. M. ZUCKER 1950 Animal protein factor and vitamin B_{12} in the nutrition of animals. Vitamins and Hormones, *8:* 2.

EXPERIMENTAL MEGALOBLASTIC ANEMIA AND SCURVY IN THE MONKEY

V. NATURE OF THE RELATION OF ASCORBIC ACID DEFICIENCY TO THE METABOLISM OF FOLIC ACID COMPOUNDS [1]

CHARLES D. MAY,[2] AGNES HAMILTON AND CHARLES T. STEWART
Departments of Pediatrics, Colleges of Medical Science, University of Minnesota, Minneapolis, and State University of Iowa, Iowa City

ONE FIGURE

(Received for publication July 7, 1952)

Once it was found that a deficiency of folic acid compounds and megaloblastic anemia developed regularly in scorbutic monkeys (May et al., '51, '52a), it became interesting to determine what influence ascorbic acid deficiency had on the metabolism of folic acid compounds.

The experiments described in this report were devised to answer the question: Does ascorbic acid deficiency disrupt the normal metabolism of folic acid at one or more points, or do some non-specific factors operating in scurvy increase the requirements for folic acid? To this end a systematic examination of the effects of ascorbic acid deficiency on each of the known phases in the metabolism of folic acid compounds was undertaken.

Present understanding of the metabolism of folic acid compounds may be outlined as follows (Welch and Heinle, '51): Primates do not appear to synthesize folic acid compounds

[1] This study was made possible by the generous support of the M and R Laboratories, Columbus, Ohio, and Wyeth, Inc., Philadelphia, Pa. Dietary supplements were provided by the Eli Lilly Co., Indianapolis, Indiana, and Distillation Products Industries, Rochester, N. Y., for which we are most grateful.

[2] Present address: Department of Pediatrics, State University of Iowa, Iowa City.

but obtain them from the diet or from the synthetic activities of the bacteria in the intestine. In both instances the folic acid usually occurs almost entirely in a conjugated form, a folic acid heptaglutamate. Presumably the folic acid must be liberated from the conjugate prior to utilization in metabolic reactions. This is accomplished by conjugases in the tissues, notably the kidney and liver. Folic acid can be converted by enzymes in the liver to a derivative, folinic acid (citrovorum factor), which may be the biologically active form. Folic acid or folinic acid plays a role in the synthesis of nucleic acids essential for normal hematopoiesis. Some workers are of the opinion that folinic acid and its conjugate are the naturally occurring forms and that folic acid is merely produced by alterations during isolation. This consideration does not require any modification of the present outline.

Consequently, it was necessary to test the effect of ascorbic acid deficiency on: (1) the bacterial synthesis of folic acid compounds, (2) the absorption of the conjugate from the intestine, (3) the liberation of the folic acid by the action of the conjugase, (4) the conversion of folic acid to folinic acid, and (5) the comparative activity of folic acid and folinic acid on megaloblastosis in scurvy.

ARRANGEMENT OF EXPERIMENTS AND METHODS

The general management, diets, and descriptions of the monkeys used in the experiments were as previously described (May et al., '51, '52a). The methods used in microbioassay of the folic acid compounds have also been published (May et al., '52a). Free folic acid (PGA) was determined in the specimens with *S. fecalis* after autoclaving, and total PGA after subsequent treatment with hog kidney conjugase. Free folinic acid (FNA) was determined with *L. citrovorum* after autoclaving the specimens without enzyme treatment. It will be recalled that "total PGA" as determined with *S. fecalis* includes folinic acid compounds as well as folic acid compounds. Free FNA may be a more accurate measure of biologically active material.

RESULTS

In the previous paper data were reported on the PGA in the diet and feces (May et al., '52a). The milk diet fed the monkeys was found to contain 2.6 μg total PGA per liter. The control animals consumed 400 to 600 ml a day, making the intake of total PGA approximately 1 μg a day. The most anorexic of the scorbutic animals drank 100 ml daily, leading to a reduction of PGA intake to about one-fourth. The PGA in milk may be predominantly in the free form (Hodson, '49).

The average daily fecal excretion of total PGA was found to be the same in the scorbutic monkeys as in the controls, about 29 μg a day. The PGA was almost entirely in the conjugated form in both cases. This indicates that ascorbic acid deficiency probably did not affect the bacterial synthesis of folic acid compounds in the intestines of these monkeys.

Absorption and utilization of folic heptaglutamate

The extent to which the conjugated PGA in the feces is available to the animals can be estimated from the following experiments:

To compare the absorption and utilization of folic acid compounds, equal amounts of folic acid in the free and conjugated forms were given to control and scorbutic monkeys, orally and intramuscularly, and the amount of free folic acid excreted in the urine was measured. It was found, by treating the urine with conjugase, that conjugated folic acid was not excreted as such in the urine even when injected intramuscularly, by either the control or the scorbutic monkeys. The data presented in table 1 indicate that orally administered free and conjugated PGA are absorbed equally well by the scorbutic and the control animals and that probably conjugated PGA is poorly absorbed by both.

The liberation of PGA from the heptaglutamate was performed normally by the scorbutic animal, as judged by the appearance of 80% or more of the intramuscularly administered conjugated PGA as free PGA in the urine. This was

TABLE 1

Utilization of folic acid (PGA) and folic heptaglutamate (PGAC) by scorbutic and control monkeys

ANIMAL NO.	DIET		EXCRETED IN URINE WITHIN 24 HOURS:	
			As free PGA	As conjugated PGA
			%	
		Controls (50 mg ascorbic acid daily)		
39	4B	500 μg PGA by mouth	17	....
30	4B	500 μg PGA intramuscularly	88	....
37	4B	500 μg PGA intramuscularly and 15 μg B_{12} intramuscularly	86	....
142	4F	200 μg conjugated PGA (75 mg PGAC concentrate [1]) by mouth	2	None [2]
142	4F	200 μg conjugated PGA (75 mg PGAC concentrate [1]) intramuscularly	39	None
		Scorbutic		
127	4D	200 μg PGA b.m.	24	None
128	4D	200 μg conjugated PGA (75 mg PGAC concentrate [1]) by mouth	4	None
129	4D	200 μg conjugated PGA (75 mg PGAC concentrate [1]) intramuscularly	83	None

[1] A concentrate of folic acid heptaglutamate derived from yeast (Greene, '49) and supplied through the courtesy of Dr. Richard D. Greene, E. R. Squibb and Sons. Assayed in our laboratory to contain 0.79% heptaglutamate. Seventy-five milligrams of this concentrate provided the equivalent of 200 μg of folic acid activity as determined by treatment with conjugase and assay with *S. fecalis*.

[2] No increase in free PGA was found in the urine after treatment with conjugase.

substantiated by the finding of: (1) the same ratio of free to total PGA in the livers of control and ascorbic acid-deficient monkeys (table 2) and, (2) that liver from scorbutic monkeys

TABLE 2

Free vs. total PGA in livers of monkeys

(Micrograms per gram, wet)

CONDITION OF MONKEYS	AVERAGE TOTAL PGA	AVERAGE FREE PGA
Adequate ascorbic acid	0.60	0.27 (45%)
Ascorbic acid deficiency	0.13	0.07 (54%)

TABLE 3

In vitro folic acid conjugase activity of monkey livers

TREATMENT OF YEAST EXTRACT [1]	μG FOLIC ACID LIBERATED PER GRAM DRIED YEAST
No treatment (free PGA)	0.6
Hog kidney "conjugase" (total PGA)	25
Control monkey liver "conjugase"	23
Scorbutic monkey liver "conjugase"	20

[1] *Preparation of yeast extract:*

Five grams of dried brewers' yeast were suspended in 50 ml distilled water, adjusted to pH 6.5, autoclaved 5 minutes at 15 pounds, cooled, centrifuged, and the supernatant solution was made up to 100 ml.

Preparation of hog kidney and monkey liver "conjugase":

The tissue was passed through a tissue press and weighed. Three parts of distilled water were added and the suspension homogenized in a Potter-Elvehjem homogenizer.

Procedure:

To 5 ml of yeast extract were added 5 ml of McIlvaine's phosphate buffer (pH 4.5) and 1 ml of the "conjugase" preparation to be tested for activity. The well-mixed suspension was then incubated for 20 hours at 37°C., placed in a boiling water bath for two minutes, neutralized with 10% NaOH, made up to a volume of 20 ml with distilled water, filtered, and diluted for assay with *S. fecalis*.

was almost as effective a source of conjugase for liberating PGA from the conjugated form in yeast as was the standard hog kidney conjugase preparation or liver from a control monkey (table 3).

Conversion of PGA to FNA

An attempt was made to ascertain the efficiency of conversion of folic acid to folinic acid by measuring the amount of free FNA excreted in the urine following an intramuscular test dose of free PGA (40 μg per kilogram). The concentration of FNA excreted by both the control and the scorbutic monkeys was too small for accurate analysis, although 25% of the administered PGA was recovered from the urine.

Another approach to detecting any effect of ascorbic acid deficiency on the metabolism of folic acid compounds, including the conversion of PGA to FNA, was to compare the accumulation of these compounds in the liver with the effect on the megaloblastic marrow when free or conjugated folic acid was administered to scorbutic megaloblastic monkeys, orally or intramuscularly. Data from this study are presented in table 4. The following considerations should be employed in evaluating the data in this table: The scorbutic megaloblastic animals were selected to be as similar as possible, so that the liver stores and marrows would be in nearly the same state before treatment. They were grouped so that test doses were given in equal amounts for the same length of time before comparing the marrows and killing the animals for liver assays. Animals to be compared were treated simultaneously with the same solutions of test materials. The diets were essentially the same, thus keeping the trace of PGA compounds obtainable from this source at a constant level.

It is evident that the scorbutic animal accumulated FNA in the liver after small doses of PGA, free or conjugated, with corresponding alleviation of the megaloblastosis in the marrow, without the aid of ascorbic acid (note animals 92 and 123). Also it may be seen that larger doses are required orally than intramuscularly. (Compare animals 120 and 119 with 92 in the case of free PGA, and 122 with 123 in the case of conjugated PGA.) The better absorption of orally administered free PGA versus conjugated PGA is not so clearly demonstrated as in table 1. It appears that scorbutic

TABLE 4

Effect of folic acid (PGA) and folic heptaglutamate (PGAC) on the megaloblastic marrow and the liver content of PGA and free folinic acid (FNA) in scorbutic monkeys [1]

	ANIMAL NO.	DIET	B_{12}	TOTAL PGA	FREE FNA	
				μg/gm wet liver		
Untreated						
Controls — normoblastic	Averages [2]	4B,4F	1.0	1.10	0.026	
Scorbutic — megaloblastic	Averages [2]	4C [2]	1.0	0.20	0.010	
Scorbutic — megaloblastic	Averages [2]	4F	0.5	0.12	0.004	
Treated 12 days:						*Marrow after treatment*
100 μg PGA by mouth	120	4F	0.5	0.24	0.009	Equivocal effect
100 μg PGA intramuscularly	92	4C [2]	1.3	0.39	0.033	Normoblastic
200 μg PGA b.m.	119	4F	0.5	0.47	0.021	Normoblastic
100 μg conjugated PGA [3] b.m.	122	4F	0.6	0.34	0.013	Megaloblastic
100 μg conjugated PGA i.m.	123	4F	0.6	0.38	0.051	Normoblastic
200 μg conjugated PGA b.m.	124	4F	0.5	0.37	0.013	Megaloblastic
200 μg conjugated PGA i.m.	117	4F	0.5	0.24	0.020	Normoblastic
Treated 7 days:						
200 μg PGA b.m.	127	4D	0.5	0.40	0.017	Normoblastic
200 μg conjugated PGA b.m.	128	4D	0.5	0.17	0.029	Equivocal effect
200 μg conjugated PGA i.m.	129	4D	0.5	0.18	0.018	Normoblastic
Treated prophylactically:						
500 μg PGA b.m. weekly	39 [4]	4B	0.6	1.69	....	Normoblastic
500 μg PGA i.m. weekly	30 [4]	4B	0.4	1.05	....	Normoblastic
500 μg PGA + 15 μg B_{12} i.m. weekly	37 [4]	4B	1.0	1.35	....	Normoblastic

[1] The monkeys were allowed to reach essentially the same degree of scurvy and megaloblastosis, treated for the same lengths of time as indicated, and then, after examining the marrow, the animals were killed and the livers assayed for comparison of the accumulations of PGA and FNA.

[2] Average values from previous report (May et al., '52a). Diet 4C was supplemented with intramuscular injections of vitamin B_{12}; all the other diets had no supplementary B_{12} or the animals received a supplement orally. Vitamin B_{12} neither prevents nor cures the megaloblastosis complicating scurvy. Controls received an abundance of ascorbic acid.

[3] A concentrate of folic acid heptaglutamate derived from yeast (Greene, '49) and supplied through the courtesy of Dr. Richard D. Greene, E. R. Squibb and Sons. Assayed in our laboratory to contain 0.79% heptaglutamate. Seventy-five milligrams of this concentrate provided the equivalent of 200 μg of folic acid activity, as determined by treatment with conjugase and assay with *S. fecalis.*

[4] Killed after signs of scurvy had been evident for three to 4 weeks.

animals absorb conjugated PGA poorly (note animals 122, 124) but no more so than control animals (see table 1). It was not possible on the basis of these data to determine whether ascorbic acid might augment the conversion of PGA to FNA *in vivo* in the monkey, as Nichol and Welch ('50) have reported it did during *in vitro* experiments with rat liver slices. It can only be asserted that an abundance of ascorbic acid does not appear to be necessary for the conversion of PGA to FNA *in vivo* in the monkey. In other studies serial observations of the marrow showed the rate of recovery from megaloblastosis to be equally rapid in scorbutic animals following large doses of PGA or ascorbic acid (Sundberg et al., '52).

Comparative activity of folic and folinic acids on megaloblastosis in scurvy

That synthetic folinic acid does not require ascorbic acid for its action has already been reported in preliminary form (May et al., '50b). Figure 1 presents data from that study in graphic form to show that the rate of disappearance of megaloblasts from the marrows of scorbutic monkeys was as rapid after 7.5-μg doses of folinic acid as it was after 750-μg doses of folic acid, and that 50-μg doses of folic acid were unable to halt the progression of the megaloblastic process. This might be interpreted as indicating that the conversion of PGA to FNA is inefficient in scurvy if FNA is considered as the biologically active form. Scurvy is the only condition in which a defective conversion of PGA to FNA has been suggested, and provides the best circumstances in which the relative biologic potency of PGA and FNA might be compared more extensively.

DISCUSSION

On the basis of these studies, the conversion of PGA to FNA is the only area in which a deficiency of ascorbic acid might be considered to affect the metabolism of folic acid

compounds. All other phases in the metabolism of these compounds were found to be unaffected in scurvy. It has been pointed out that whereas the efficiency of conversion of PGA to FNA may be impaired in scurvy, the evidence for this in these studies in the monkey was scanty. The conversion proceeded sufficiently well in scurvy to permit small doses

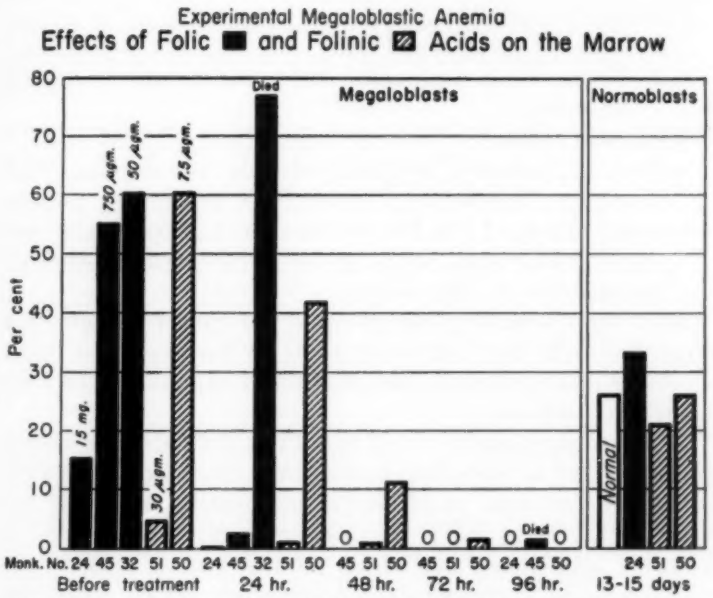


Fig. 1 Comparison of effects of folic and folinic acids on megaloblastic marrows in scorbutic monkeys. (From data in study by May, Sundberg and Schaar, '50b). The symbol O indicates that the marrow was not examined at the corresponding time interval.

of PGA to be used effectively for correction of the deficiency of FNA and elimination of the megaloblastosis.

It therefore seems necessary to seek an explanation other than ascorbic acid deficiency to account for the development of so profound a deficiency of PGA and FNA as occurs in scurvy. It would seem more reasonable to attribute this de-

ficiency to inability to meet an increased requirement for PGA in scurvy, due to greater use or wastage of PGA because of non-specific factors operating in scurvy. This may be expressed as follows:

Increased daily requirement for PGA in scorbutic monkeys on milk diets

Potentially available PGA:

1. Milk diet, 2 μg free PGA.
2. Feces, 30 μg conjugated PGA which may be poorly absorbed.

With this intake:

Controls, ascorbic acid-adequate, do not develop PGA deficiency. Scorbutics develop PGA deficiency. Supplements of 5 μg of free PGA daily cannot prevent deficiency (May et al., '50a), but 70 μg daily will prevent deficiency (animals 30, 37, 39, table 4).

Thus the scorbutic monkeys could not obtain enough PGA from the milk diet and their feces to maintain the tissue stores at a normal level, but required a supplement of PGA to prevent deficiency.

It is also pertinent to recall that PGA deficiency sufficiently severe to lead to megaloblastosis rarely developed until signs of scurvy had been manifest in the monkey for about two weeks (May et al., '51). It was not merely a deficiency of ascorbic acid alone, but actual clinical scurvy with its attendant tissue hemorrhages and pathology, which caused the difficulty. It can be seen in table 5 that the stores of ascorbic acid declined rapidly on the deficient diet, reaching a level nearly as low as that found in scurvy in about 30 days, though scurvy did not appear for another 50 to 60 days. The levels of folic acid compounds fell more gradually until signs of scurvy appeared, and then a sharp decrease in PGA and particularly in FNA occurred.

This raises the question: What causes the sharp decline of PGA compounds in the tissues upon the advent of scurvy? At present we suspect that scurvy is complicated by a defi-

ciency of PGA and FNA and by megaloblastosis primarily because of non-specific factors in scurvy which cause increased use or loss of PGA, rather than because of any specific role of ascorbic acid in the metabolism of folic acid compounds. The diffuse pathology of scurvy may be considered a form of "stress." The effect of "stress" situations on the tissue stores of folic acid compounds has been under investigation (May et al., '52b); infection causes marked depletion of tissue stores of folic acid compounds, even when the concentration of ascorbic acid in the tissues is normal.

TABLE 5

Relation between development of ascorbic acid deficiency and folic acid deficiency

ANIMALS	ASCORBIC ACID	TOTAL PGA	FREE FNA	MARROW
		μg/gm wet liver		
Controls (average)[1]	129	1.10	0.026	Normoblastic
No ascorbic acid				
30 days (No. 157) biopsy	12	0.96	0.018	Normoblastic
82 days (No. 157) biopsy	6	0.52	0.023	Normoblastic
101 days (No. 157) scurvy 7 days	4	0.23	0.003	Megaloblastic

[1] From tables 2 and 4 in previous paper (May et al., '52a).

It is possible that vitamin B_{12} is involved in the metabolism of folic acid compounds. Our scorbutic monkeys were not markedly deficient in vitamin B_{12}. If vitamin B_{12} requires ascorbic acid to function normally, then the metabolism of folic acid compounds might be disturbed in scurvy in this indirect manner. A suggestion to an obstetrical colleague, that B_{12} might be more effective in the megaloblastic anemia of pregnancy if ascorbic acid were given first, led to the finding that this was the case (Holly, '51). The interrelations of B_{12}, ascorbic acid and folic acid will have to be studied by means of animals made deficient in B_{12} as well as ascorbic acid.

CONCLUSIONS

As a result of a systematic study of the effect of ascorbic acid deficiency on the metabolism of folic acid compounds it was concluded that:

1. The conversion of folic acid to folinic acid *in vivo* may be less efficient in scurvy, but ascorbic acid is not necessary for this conversion to take place. All other phases of the metabolism of folic acid compounds tested were found to proceed normally in scurvy; ascorbic acid is not required.

2. Ascorbic acid deficiency was not accompanied by a marked deficiency of folic acid compounds until signs of scurvy had become well advanced.

3. The severe deficiency of folic acid compounds which occurred regularly as a complication of scurvy in monkeys fed milk diets was probably due to non-specific factors operating in scurvy.

4. The net effect of scurvy was to cause increased requirements for folic acid compounds which could not be met by the supply of these compounds obtainable from the milk diet and the feces.

LITERATURE CITED

GREENE, R. D. 1949 The extraction of folic (pteroylglutamic) acid conjugate from yeast. J. Biol. Chem., *179:* 1075.

HODSON, A. Z. 1949 The microbiological assay of "folic acid" as applied to milk. J. Nutrition, *38:* 25.

HOLLY, R. G. 1951 Megaloblastic anemia in pregnancy. Remission following combined therapy with ascorbic acid and vitamin B_{12}. Proc. Soc. Exp. Biol. Med., *78:* 238.

MAY, C. D., A. HAMILTON AND C. T. STEWART 1952a Experimental megaloblastic anemia and scurvy in the monkey. IV. Vitamin B_{12} and folic acid compounds in the diet, liver, urine and feces and effects of therapy. Blood, *7:* 978.

MAY, C. D., E. N. NELSON, C. U. LOWE AND R. J. SALMON 1950a Pathogenesis of megaloblastic anemia in infancy. An interrelationship between pteroylglutamic acid and ascorbic acid. Am. J. Dis. Child., *80:* 191.

MAY, C. D., C. T. STEWART, A HAMILTON AND R. J. SALMON 1952b Infection as a cause of folic acid deficiency and megaloblastic anemia: experimental induction of megaloblastic anemia by turpentine abscess. Ibid., in press.

MAY, C. D., R. D. SUNDBERG AND F. SCHAAR 1950b Comparison of effects of folic acid and folinic acids in experimental megaloblastic anemia. J. Lab. Clin. Med., *36:* 963.

MAY, C. D., R. D. SUNDBERG, F. SCHAAR, C. U. LOWE AND R. J. SALMON 1951 Experimental nutritional megaloblastic anemia: relation of ascorbic acid and pteroylglutamic acid. I. Nutritional data and manifestations of animals. Am. J. Dis. Child., *82:* 282.

NICHOL, C. A., AND A. D. WELCH 1950 Synthesis of citrovorum factor from folic acid by liver slices; augmentation by ascorbic acid. Proc. Soc. Exp. Biol. Med., *74:* 52.

SUNDBERG, R. D., F. SCHAAR AND C. D. MAY 1952 Experimental nutritional megaloblastic anemia. II. Hematology. Blood, in press.

WELCH, A. D., AND R. W. HEINLE 1951 Hematopoietic agents in macrocytic anemias. Pharm. Rev., *3:* 345–411.

EFFECTS OF DIETARY AUREOMYCIN UPON THE INTESTINAL MICROFLORA AND THE INTESTINAL SYNTHESIS OF VITAMIN B_{12} IN THE RAT [1]

K. R. JOHANSSON, G. E. PETERSON AND ELLIOT C. DICK

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis

FOUR FIGURES

(Received for publication July 24, 1952)

A number of investigations support the hypothesis that antibiotics stimulate the growth of young poultry and swine as a result of alterations of the "normal" intestinal microflora (Elam et al., '51a; Kratzer et al., '51; March and Biely, '52; Romoser et al., '52; Sieburth et al., '51; Williams et al., '51). In an attempt to elucidate further the mechanism concerned, Elam et al. ('51b) postulated, from results with the chicken, that the antibiotic molecule, or a fragment thereof, is utilized as a metabolite by the host. Other evidence obtained with the chicken (Dornbush et al., '51; McGinnis et al., '50; Whitehill et al., '50) and the rat (Cravioto-Munoz et al., '51) has not indicated the existence of such a mechanism. Studies with poultry (Oleson et al., '50; Stokstad and Jukes, '51), swine (Edwards et al., '51) and rats (Cravioto-Munoz et al., '51; Oleson et al., '50; Waisman et al., '51) indicate that dietary antibiotics exert a "vitamin B_{12}-sparing action," presumably as a consequence of obscure changes in the intestinal flora. It now appears that B_{12} is not the only nutrilite concerned in the growth response to antibiotics (Biely and March,

[1] This investigation was supported in part by Research Grant 2995 from the National Institutes of Health, Public Health Service.

'51; Lih and Baumann, '51; Linkswiler et al., '51; Sauberlich, '52). Most workers believe that effects on intestinal bacteria are partially or wholly responsible for the growth response of some animals fed antibiotics. Since information concerning the nature of these changes is limited, it seemed desirable to study, in some detail, the influence of aureomycin upon the intestinal microflora of the rat, and concurrently to explore the interrelationships between the intestinal biosynthesis of vitamin B_{12} and dietary aureomycin. This is the first report of these investigations.

EXPERIMENTAL METHODS

Twenty-eight male weanling rats of the Sprague-Dawley strain, from 40 to 50 gm in weight, were housed by pairs in wide-mesh, wire-bottomed battery cages. Seven groups of 4 rats each received one of the following diets during a 30-day period: unsupplemented basal; basal + B_{12} (crystalline vitamin B_{12},[2] 20 μg/kg); basal + aureomycin[3] (aureomycin hydrochloride, 100 p.p.m.); basal + B_{12} + aureomycin; basal + methionine (DL-methionine, 0.6%); stock ration[4]; and stock + aureomycin (100 p.p.m.). These rations — furnished in feeding cups designed to minimize fecal contamination — and water were supplied ad libitum. The temperature of the room was controlled between 24° and 26°C. The animals were weighed several times a week and a record was kept also of the amount of food consumed.

The purified basal ration, deficient in vitamin B_{12} and suboptimum in methionine, was of the following composition: "alpha protein" (isolated soybean protein), 21.8 parts; sucrose, 50 parts; L-cystine, two-tenths part; "salts 4" (Hegsted et al., '41, modified to contain one-tenth the amount of manganese), 4 parts; hydrogenated vegetable fats,[5] 6 parts; cel-

[2] Merck.

[3] Preliminary experiments indicated that a dietary level of 50 or 100 p.p.m. aureomycin was as stimulating to growth as higher levels (up to 3,000 p.p.m.); lower levels did not give as good a response.

[4] Rockland Rat Diet.

[5] Crisco.

lulose ("alphacel"),[6] 5 parts; sucrose-vitamin mixture (thiamine chloride, 5 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 5 mg; calcium pantothenate, 25 mg; niacin, 20 mg; *i*-inositol, 200 mg; choline chloride, 1,000 mg; folic acid, 0.25 mg; para-aminobenzoic acid, 50 mg; menadione, 2.5 mg; biotin, 0.1 mg; sucrose to a total of 100 gm), 10 parts; corn oil-vitamin mixture (oleum percomorphum, 0.6 gm; d-alpha-tocopherol, 0.25 gm; corn oil to a total of 100 gm), three parts.

Qualitative and quantitative microflora studies were carried out for each group on pooled fecal dropping samples collected on non-absorbent paper. After 5 weeks, contents from the cecum and ileum of two rats at a time were pooled and the microflora analyzed immediately. The types of microbial counts performed on these specimens and the media employed are listed in table 1. Each sample was diluted 1:100 in bottles containing sterile tap water and glass beads and, following a brief soaking period, was shaken vigorously for 5 minutes. Further serial decimal dilutions were made in sterile tap water for quantitation in various media. All plate and shake-tube counts were prepared in duplicate and, for dilution counts, 5 tubes were inoculated per dilution and a "most probable numbers table" (Am. Pub. Health Assn., '48) employed in estimating the original population.

Assays of fecal material for vitamin B_{12} were carried out with a mutant culture of *Escherichia coli* (strain 113-3), kindly furnished by Dr. Bernard D. Davis, according to the method of Johansson ('51). Digestion, when desired, was accomplished with trypsin at 37°C. for 24 hours under toluene. Crystalline vitamin B_{12}[7] was employed as a standard.

RESULTS

Animal growth

Figure 1 shows that, under the conditions of this experiment, growth was not stimulated markedly by aureomycin

[6] Nutritional Biochemicals Corp.

[7] See footnote 2, p. 136.

unless vitamin B_{12} was present in the purified ration. One rat fed the unsupplemented basal diet (insufficient in methionine and deficient in B_{12}) died and three were sacrificed on the 10th day when symptoms (bloody nasal discharge and respiratory difficulty), which may have resulted from a methionine deficiency, were apparent. The addition of aureomycin to the basal mixture, however, delayed the onset of this deficiency until the 19th day. Vitamin B_{12} and methionine were equally beneficial to growth when added to the deficient basal ration.

TABLE 1

Microbiological methods used for the estimation of intestinal flora changes

TYPE OF COUNT OR ORGANISM	MEDIUM USED	INCUBATION	REFERENCE
Aerobic plate count	Tryptone-glucose-yeast extract agar	37°C., 48 hr.	Johansson et al. ('48)
Anaerobic plate count	Tryptone-glucose-yeast extract agar	Atm. of 90% H_2 + 10% CO_2 at 37°C., 48 hr.	Johansson et al. ('48)
Lactobacilli by shake-tube count	Carrot-liver extract agar modified with 2.5% Na acetate	37°C., 48 hr.	Garey et al. ('41)
Aureomycin-resistant aerobes by plate count	Tryptone-glucose-yeast extract agar + 10 μg aureomycin/ml	37°C., 48 hr.	Johansson et al. ('48)
Coliform organisms by plate count	Violet red bile agar	37°C., 48 hr.	Difco Laboratories, Detroit
Aureomycin-resistant coliforms by plate count	Violet red bile agar + 10 μg aureomycin/ml	37°C., 48 hr.	Difco Laboratories, Detroit
Enterococci by 5-tube dilution count	"SF" medium	45°C., 72 hr.	Hajna and Perry ('43); Difco Laboratories
"Total" no. by 5-tube dilution count	"Fluid thioglycolate medium"	37°C., 48 hr.	Baltimore Biol. Lab., Baltimore
Yeasts by plate count	Acidified potato-dextrose agar	Room temp., 5 days	Am. Pub. Health Assn. ('48); Difco Laboratories

However, aureomycin produced a pronounced growth response when incorporated in the vitamin B_{12}-supplemented diet. Animals fed a ration containing both B_{12} and aureomycin grew nearly as rapidly during the first two weeks as those receiving the natural diet, but thereafter growth slackened. Supplementation of the natural diet with aureomycin resulted in a slight growth stimulation which lasted but two weeks. The efficiency of food utilization (weight gained: food consumed)

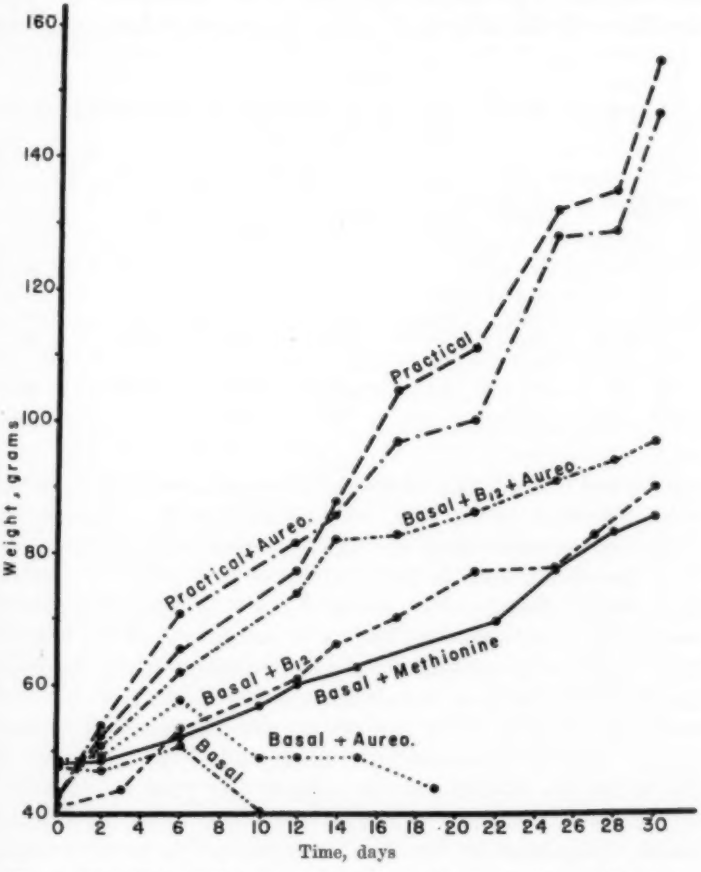


Fig. 1 The effect of dietary aureomycin on the growth of weanling rats.

was not affected by aureomycin and was greater with the stock diet than with any of the purified mixtures. (Note: This study was concerned primarily with the microflora and vitamin B_{12} activity in the rat's intestine. These objectives precluded the use of larger numbers of animals and therefore no attempt was made to treat the data on weight changes and food utilization statistically.)

Table 2 shows the vitamin B_{12} content of successive composite samples of fecal droppings. The feces were not predigested and the recorded values represent what might be

TABLE 2

The vitamin B_{12} content (mμg per gram, wet weight) of fecal droppings from rats fed various rations

NO. DAYS ON RATION	RATION					
	Basal	Basal + aureo.	Basal + B_{12}	Basal + B_{12} + aureo.	Stock	Stock + aureo.
1					2,700	1,110
2	950	190				
4	600	950	850		800	1,500
5	760	300	600	900	2,800	1,700
6	310	220				
16			1,290	1,030	1,790	590
20			620	620		

considered "free" B_{12}. Aureomycin appeared to have no appreciable effect on the fecal level of vitamin B_{12}. Vitamin B_{12} assays were made upon a single, undigested, pooled sample from the cecum and the ileum of those rats given the following rations: basal + B_{12}; basal + B_{12} + aureomycin; stock; and stock + aureomycin. The same magnitude of B_{12} activity was found in the cecum as in fecal droppings, while the ileum contained one-fifth to one-sixth as much B_{12} as feces or cecal contents. Cecal levels of vitamin B_{12} were unaffected by aureomycin. However, aureomycin lowered the concentration of B_{12} in the ilea of rats fed the natural diet (260 vs. < 10 mμg B_{12}/gm, wet weight) but not of those ingesting the purified ration. Digestion of intestinal samples prior to performing

the assay gave slightly higher calculated levels of B_{12} in the cecum but was without effect on ileal samples.

Intestinal flora

Data from the microbiological analyses of the fecal droppings are depicted in figures 2 and 3. Each pair of bars show the logarithm of the number of bacteria per gram (wet weight) obtained with a particular medium as indicated along the abscissa and the bacteria are in the same order, from left to right, as those listed in table 1. The bars filled in with diagonal stripes represent the average of counts made on 4 separate pooled samples from rats fed aureomycin-free diets (the presence or absence of vitamin B_{12} in the diet did not appear to affect the fecal flora, hence each bar in fig. 2 is an average count from two ration gorups). The dot-filled bars signify average fecal counts of rats ingesting the aureomycin-containing rations. Since it was of interest to determine the rate at which changes were induced in the intestinal microflora by the antibiotic, the latter bars are broken at the top into steps. The first step on the left indicates the population estimated 12 hours after the experimental period began, while the remaining steps represent, in order, results of the 36th-, 84th-, and 144th-hour samples. In three cases the top of the first step is not filled in because the determinations were not made. Yeast counts are not included in these or subsequent graphs because no striking effects on these organisms could be detected. These data reveal that aureomycin had little effect on "total" numbers of bacteria in terms of thioglycolate dilution or aerobic and anaerobic plate counts. Total numbers of coliform bacteria in the feces appeared to be unaffected when aureomycin was incorporated in the purified diets, but its addition to the stock diet resulted in an increase of these organisms. Otherwise, alterations of the fecal flora by the antibiotic were similar in either ration group.

Results of an analysis of variance of these data are assembled in table 3. The only fecal counts which aureomycin did not alter significantly were those of coliforms and "total"

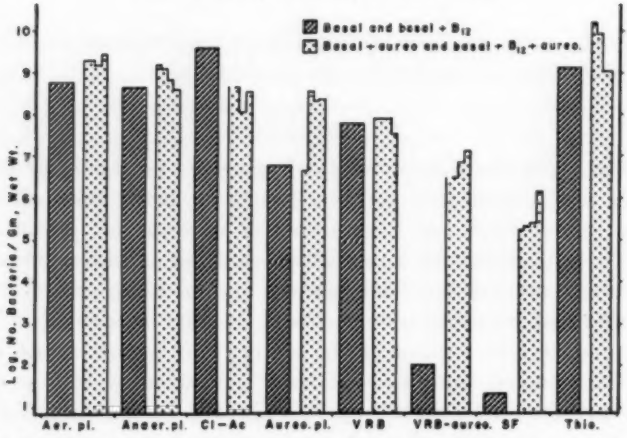


Fig. 2 Bacterial counts of feces from rats fed purified aureomycin-containing rations.

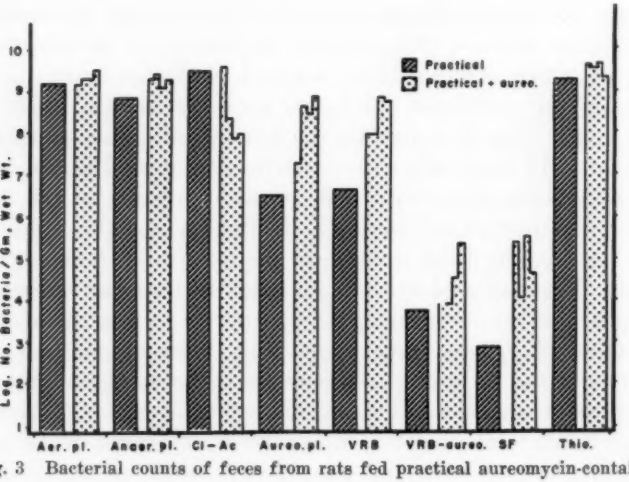


Fig. 3 Bacterial counts of feces from rats fed practical aureomycin-containing rations.

KEY TO FIGURES 2, 3 AND 4

Key to media (see table 1): "Aer. pl." — aerobic plate count; "Anaer. pl." — anaerobic plate count; "Cl-Ac" — lactobacillus count with carrot-liver-acetate agar; "Aureo. pl." — aerobic plate count of aureomycin-resistant bacteria; "VRB" — coliform plate count with violet red bile agar; "VRB-aureo." — plate count of aureomycin-resistant coliforms; "SF" — enterococcus dilution counts with "SF" medium; "Thio." — total dilution count with "Fluid Thioglycolate Medium."

aerobes from animals fed the purified diets. Enterococcus enumerations from animals on the practical ration were not treated statistically because of insufficient replication. Least significant difference (LSD) determinations revealed that aureomycin brought about statistically-significant changes in the fecal flora within 12 or 36 hours except among aureomycin-resistant coliforms, which did not increase significantly until 144 hours had elapsed. It is interesting to note, however, that aureomycin increased significantly the "total" numbers of aureomycin-resistant aerobes in the fecal droppings within 36 hours ($p = <0.01$). Aureomycin caused the following obvious changes in the fecal microflora: a decrease in numbers of lactobacilli [8] and a dramatic increase in numbers of aureomycin-resistant bacteria and enterococci. These effects usually occurred within 12 to 36 hours and were of a magnitude of from 10- to over 10,000-fold.

The bacterial counts of ileal and cecal contents obtained at the end of the experiment are assembled in figure 4. Each bar indicates the average of two pooled samples per intestinal level from each dietary group (except basal + methionine). This graph is similar to the two previous figures except that data for all rations are grouped together for comparative purposes. The left-hand step of each bar indicates the average population level in the ileum and the other step represents the average cecal value. Because of the lack of sufficient numbers of samples, these data were not evaluated statistically. It can be seen that the changes which aureomycin produced in the microflora of the intestines were similar to those which occurred in the feces. Aureomycin encouraged proliferation in the ileum and cecum of coliforms, aureomycin-resistant bac-

[8] It should be emphasized that the carrot-liver medium containing 2.5% sodium acetate was selective for the gram-positive bacteria and that its use resulted in a good estimate of the lactobacillus population because other gram-positive bacteria were not present in the high dilutions necessary to enumerate this group. All isolates obtained from this medium, except in lower dilutions, were found to be characteristic of the genus *Lactobacillus*. A new medium (Rogosa et al., '51), highly selective for lactobacilli, is being employed successfully in present investigations of this nature.

TABLE 3

Summary of statistical analysis of bacterial counts (number per gram, wet weight) from fecal droppings of weanling rats fed aureomycin

TYPE OF BACTERIUM COUNTED	RATION	HOURS AFTER START OF AUREOMYCIN FEEDING					STATISTICS [1]		
		0	12	36	84	144	F value	LSD 5%	LSD 1%
"Total" aerobes	Purified	9.6	45.3	22.4	16.1	27.8	1.5 (ns)	..	..
($\times 10^8$)	Stock	13.4	13.6	18.6	19.5	36.0	36.2**	4.3	7.1
Lactobacillus	Purified	43.0	...	2.4	2.8	3.8	37.1**	11.5	16.5
($\times 10^8$)	Stock	45.0	42.5	23.0	0.9	1.0	179.6**	9.0	14.9
Aureo-resistant	Purified	5.1	4.9	370.0	192.5	266.3	16.7**	119.6	167.7
aerobes ($\times 10^6$)	Stock	4.6	21.9	78.0	219.5	700.0	336.4**	43.8	72.7
Coliforms	Purified	69.3	80.0	80.0	81.8	37.8	1.6 (ns)	..	..
($\times 10^6$)	Stock	6.7	80.0	80.0	670.0	625.0	396.5**	52.5	87.0
Aureo-resistant	Purified	0.0	...	24.3	70.3	380.0	6.5**	254.9	366.6
coliforms ($\times 10^5$)	Stock	0.06	...	0.08	0.4	2.4	10.8*	1.2	..
Enterococcus	Purified	0.1	90.0	90.0	140.0	825.0	462.5**	87.0	144.3
($\times 10^3$)									

[1] F = F value as calculated by analysis of variance (* — significant at the 5% level of probability; ** — significant at the 1% level; (ns) — differences not significant). LSD = least significant difference value (at 5 and 1% levels of probability).

teria and enterococci, although the increase in numbers of enterococci was not nearly as pronounced as that found in expelled feces. Lactobacilli were suppressed and it would appear from the thioglycolate dilution counts that aureomycin lowered, slightly, the "total" bacterial population in the ileum.

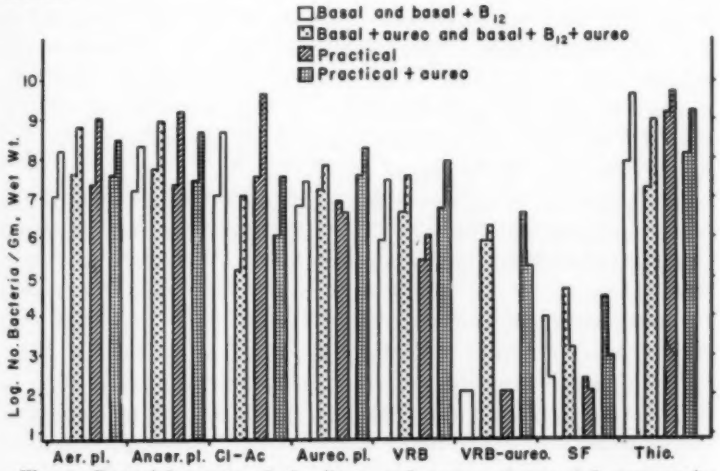


Fig. 4 Bacterial counts of the ileum and cecum of rats fed aureomycin-containing rations.

DISCUSSION

It is obvious that the purified ration employed in these studies contained an inadequate amount of methionine, and that neither vitamin B_{12} nor aureomycin was able to compensate entirely for the deficiency. However, when both of the latter factors were added to the basal mixture, a synergistic effect on growth occurred. The inability of supplementation with both B_{12} and aureomycin to promote growth at a rate comparable to that observed in those rats fed the stock diet is probably a reflection of the low methionine content of the basal ration. Nevertheless, a "methionine-sparing action" by B_{12} and aureomycin may be inferred on the basis of this investigation. The inability of aureomycin to stimulate

the growth of rats fed the natural ration might be interpreted to mean that healthy, disease-free animals given access to a well-balanced diet cannot improve their nutritional status if environmental factors are otherwise favorable. It would seem that a multiplicity of nutrients are related to this problem, since the nature of the dietary protein (Vijayaraghavan et al., '52) and carbohydrate (Monson et al., '52; Romoser et al., '52; Sauberlich, '52) affects the growth responses obtained with antibiotics. Likewise, vitamins other than B_{12} appear to play an important role in this phenomenon (Biely and March, '51; Lih and Baumann, '51; Linkswiler et al., '51; Sauberlich, '52; Waisman et al., '51).

The presence of appreciable quantities of vitamin B_{12} in the cecal contents and feces of rats maintained on the unaltered basal diet confirms the reports of others (Davis and Chow, '51; Hartman et al., '51; Lewis et al., '52) that this factor is synthesized in the intestine. It has been reported that only a small fraction of dietary B_{12} is absorbed (Chow et al., '51) and some even degraded (Barbee and Johnson, '51). In this study, however, its synthesis (presumably by intestinal organisms) seemed to be of some benefit. Since B_{12} has been shown to be absorbed rapidly from the substrate by certain bacteria (Davis et al., '52; Oginsky, '52), it is possible that dietary aureomycin may inhibit such an action, particularly in the upper reaches of the gastrointestinal tract where the antibiotic activity would be maximum. Thus, the efficiency of assimilation of dietary B_{12} by the host may be increased by aureomycin. It then becomes a question of the availability to the host of the B_{12} synthesized in the intestinal tract, the types of B_{12} in the gastrointestinal tract (Lewis et al., '52) and the involvement of Castle's "intrinsic factor."

The results of this investigation indicate that dietary aureomycin brings about a rapid and drastic alteration of the intestinal microflora, particularly within the coliform, enterococcus and lactobacillus groups of bacteria. Since coliform bacteria are able to synthesize most growth factors which they require (including vitamin B_{12}), and lactobacilli demand

a wide variety of preformed vitamins, amino acids, and so forth, the shift in the relative numbers of these organisms in favor of the coliforms may reduce the total amounts of nutrilites bound within nutritionally fastidious cells and stimulate the synthesizing flora (Johansson and Sarles, '49). The decrease in lactobacilli was compensated for by a rise in enterococci, which require fewer growth factors than the former. Thus, the pronounced reapportionment of intestinal bacteria found to occur under the influence of dietary aureomycin may have a profound effect on the competitive equilibrium which exists both within the intestinal flora and between these organisms and the host for nutrients. The effect of an antibiotic upon the intestine's ecological balance is clearly demonstrated by the report (Sieburth et al., '52) that terramycin reduced the numbers of "*Proteus* antagonists" (coliforms and enterococci) concomitant with a rise in *Proteus* spp. in the feces of turkeys without altering the number of "total aerobes." During preliminary stages of this work, it was observed that "total" counts were not altered in the presence of dietary levels of aureomycin as high as 300 to 3,000 p.p.m. This is an interesting contrast to studies made upon humans, although the efficacy of antibiotics in lowering the intestinal population in man is controversial (Metzger et al., '52). In addition to effects that antibiotic-induced alterations of the intestinal flora seem to exert upon dietary factors, such flora changes may benefit the host by minimizing the formation of microbial toxins. Evidence which supports this contention is scant (Larson and Carpenter, '52; Sieburth et al., '51; Williams et al., '51). Undoubtedly many changes in the intestinal flora occur which cannot be detected or measured because of the inadequacy of existing microbiologic methods and the magnitude of the task.

One of the most striking findings was the rapid development of large numbers of aureomycin-resistant bacteria in the gastrointestinal tract. Drug-fastness in the intestinal flora has been reported by others (Elam et al., '51a, b; Starr and Reynolds, '51) and may be noteworthy because of the

altered nutritional status of bacterial cultures when resistance to penicillin (Gale, '48) or streptomycin (Sevag and Rosanoff, '52) is induced. Similar physiological changes may occur among the intestinal bacteria when antibiotics are administered which could conceivably modify growth.

Since dietary antibiotics do alter the intestinal flora, the ensuing growth response may be a reflection of such an action. However, the report by Elam and co-workers ('51b) lends support to the proposal that antibiotics or their degradation products stimulate growth by participating directly in metabolic pathways within the body of the host. If this be true, changes in the intestinal microflora may be inconsequential or of secondary importance. Their evidence was based upon an inability to detect changes in the flora of fecal droppings from chicks given bacitracin or autoclaved penicillin, either parenterally or orally. However, it is conceivable that changes in the fecal flora occurred for which no tests were made. Furthermore, the microflora may have been influenced at higher levels of the gastrointestinal tract; e.g., the small intestine or crop.[9]

Theories which might explain how some bacteriostatic agents operate in evoking a growth response have been fairly exhaustive, and most of them implicate intestinal microorganisms. Studies with germ-free animals would aid immeasurably in resolving this dilemma.[10] Since so little basic knowledge is available concerning the interrelationships between various intestinal organisms and the host (Johansson and Sarles, '49), the discovery of a valid mechanism by which antibiotic agents operate in stimulating growth must await more penetrating investigations. The mere demonstration of changes in the intestinal flora leaves much to speculation. A rational approach, however, may evolve out of such findings.

[9] One of the authors (K.R.J.) successfully demonstrated considerable antibiotic activity in jejunal and duodenal juices of a patient receiving aureomycin intravenously (1.1 gm in three injections over a 24-hour period), as determined by the paper disc-agar plate method. Stools were not tested.

[10] At a colloquium held recently, Reyniers and his colleagues ('52) reported antibiotics to exert no influence on the growth of germ-free chickens and turkeys.

SUMMARY

The feeding of aureomycin stimulated the growth of weanling male albino rats maintained on purified rations limiting in methionine or vitamin B_{12}, or both, but was without effect on the growth of rats fed a practical diet.

Dietary aureomycin was found to cause a rapid, statistically significant change within the intestinal microflora of the rat, particularly among the coliform, enterococcus and lactobacillus groups of bacteria. A large portion of the flora, notably coliforms, developed resistance to aureomycin. These changes occurred in fecal droppings as well as in the cecum and ileum.

Vitamin B_{12} was synthesized in the intestines of rats fed rations (with or without aureomycin) deficient in this factor. No conclusive effect of aureomycin on the concentration of vitamin B_{12} in the intestinal tract was demonstrated.

ACKNOWLEDGMENTS

We are indebted to Dr. Max O. Schultze, Division of Agricultural Biochemistry, University of Minnesota, St. Paul, for his numerous helpful suggestions while carrying out this investigation and preparing this manuscript.

Aureomycin hydrochloride and most of the vitamins and amino acids required were generously furnished through the courtesy of Dr. Stanton M. Hardy, Lederle Laboratories, Pearl River, N. Y. Doctor Karl Folkers, Merck and Co., Rahway, N. J., kindly supplied the crystalline vitamin B_{12}. The soybean "alpha" protein was supplied, in part, by Mr. W. M. Bain, The Glidden Co., Soya Products Division, Chicago, Ill.

LITERATURE CITED

AMERICAN PUBLIC HEALTH ASSOCIATION 1948 Standard Methods for the Examination of Dairy Products, 9th ed.

BARBEE, K. W., AND B. C. JOHNSON 1951 Metabolism of radioactive vitamin B_{12} by the rat. Proc. Soc. Exp. Biol. Med., *76:* 720.

BIELY, J., AND B. MARCH 1951 The effect of aureomycin and vitamins on the growth rate of chicks. Science, *114:* 330.

CHOW, B. F., C. ROSENBLUM, R. H. SILBER, D. T. WOODBURY, R. YAMAMOTO AND C. A. LANG 1951 Oral administration of vitamin B_{12} containing Co^{60} to rats. Proc. Soc. Exp. Biol. Med., *76:* 393.

CRAVIOTO-MUNOZ, J., H. G. PONCHER AND H. A. WAISMAN 1951 Vitamin B_{12}-sparing action of aureomycin in the rat. Ibid., *77:* 18.

DAVIS, R. L., AND B. F. CHOW 1951 Content of radioactive vitamin B_{12} in the feces of rats fed Co^{60} and aureomycin. Ibid., *77:* 218.

DAVIS, R. L., L. L. LAYTON AND B. F. CHOW 1952 Uptake of radioactive vitamin B_{12} by various microorganisms. Ibid., *79:* 273.

DORNBUSH, A. C., J. J. OLESON, A. L. WHITEHILL AND B. L. HUTCHINGS 1951 Biological activity of alkali treated aureomycin. Ibid., *76:* 676.

EDWARDS, H. M., T. J. CUNHA, G. B. MEADOWS, G. B. SHAWVER AND A. M. PEARSON 1951 Effect of APF in supplying multiple factors for the pig. Ibid., *76:* 173.

ELAM, J. F., L. L. GEE AND J. R. COUCH 1951a Effect of feeding penicillin on the life cycle of the chick. Ibid., *77:* 209.

———— 1951b Function and metabolic significance of penicillin and bacitracin in the chick. Ibid., *78:* 832.

GALE, E. F. 1948 The nitrogen metabolism of gram positive bacteria. Bull. Johns Hopkins Hosp., *83:* 119.

GAREY, J. C., E. M. FOSTER AND W. C. FRAZIER 1941 The bacteriology of brick cheese. I. Growth and activity of starter bacteria during manufacture. J. Dairy Sci., *24:* 1015.

HAJNA, A. A., AND C. A. PERRY 1943 Comparative study of presumptive and confirmative media for bacteria of the coliform group and for fecal streptococci. Am. J. Public Health, *33:* 550.

HARTMAN, A. H., L. P. DRYDEN AND C. A. CARY 1951 The effect of riboflavin on the bacterial synthesis of vitamin B_{12}-active material in the rat. Arch. Biochem. Biophys., *34:* 324.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

JOHANSSON, K. R. 1951 A turbidimetric assay for vitamin B_{12} employing a mutant of *Escherichia coli*. Bact. Proc., 146.

JOHANSSON, K. R., AND W. B. SARLES 1949 Some considerations of the biological importance of intestinal microorganisms. Bact. Rev., *13:* 25.

JOHANSSON, K. R., W. B. SARLES AND S. K. SHAPIRO 1948 The intestinal microflora of hens as influenced by various carbohydrates in a biotin-deficient ration. J. Bact., *56:* 619.

KRATZER, F. H., C. R. GRAU, M. P. STARR AND D. M. REYNOLDS 1951 Growth-promoting activities of antibiotics and yeast cultures for chicks and turkey poults. Fed. Proc., *10:* 386.

LARSON, N. L., AND L. E. CARPENTER 1952 Effect of antibacterial substances on fecal clostridia populations. Proc. Soc. Exp. Biol. Med., *79:* 227.

LEWIS, U. J., D. V. TAPPAN AND C. A. ELVEHJEM 1952 A new and biologically different form of vitamin B_{12}. J. Biol. Chem., *194:* 539.

LIH, H., AND C. A. BAUMANN 1951 Effects of certain antibiotics on the growth of rats fed diets limiting in thiamine, riboflavin, or pantothenic acid. J. Nutrition, *45:* 143.

LINKSWILER, H., C. A. BAUMANN AND E. E. SNELL 1951 Effect of aureomycin on the response of rats to various forms of vitamin B_{12}. Ibid., *43:* 565.

MARCH, B., AND J. BIELY 1952 The effect of feeding aureomycin on the bacterial content of chick feces. Poultry Sci., *31:* 177.

MCGINNIS, J., L. R. BERG, J. R. STERN, R. A. WILCOX AND G. E. BEARSE 1950 Effect of aureomycin and streptomycin on the growth of chicks and turkey poults. Ibid., *29:* 771.

METZGER, W. I., L. T. WRIGHT, R. F. MORTON, J. C. DI LORENZO AND M. MARMELL 1952 Antibacterial action of oral aureomycin on the contents of the colon of man. Antibiotics Chemother., *2:* 91.

MONSON, W. J., L. S. DIETRICH AND C. A. ELVEHJEM 1952 The effect of different carbohydrates and antibiotics on the growth of chicks and the storage of vitamins. J. Nutrition, *46:* 411.

OGINSKY, E. L. 1952 Uptake of vitamin B_{12} by *Escherichia coli*. Arch. Biochem. Biophys., *36:* 71.

OLESON, J. J., B. L. HUTCHINGS AND A. R. WHITEHILL 1950 The effect of feeding aureomycin on the vitamin B_{12} requirement of the chick. Arch. Biochem., *29:* 334.

REYNIERS, J. A., T. D. LUCKEY AND H. A. GORDON 1952 Studies on the growth effect of antibiotics in germ-free animals. A colloquium held June 4, 1952, Univ. of Notre Dame.

ROGOSA, M., J. A. MITCHELL AND R. F. WISEMAN 1951 A selective medium for the isolation and enumeration of oral and fecal lactobacilli. J. Bact., *62:* 132.

ROMOSER, G. L., M. S. SHORB, G. F. COMBS AND M. J. PELCZAR, JR. 1952 Effect of antibiotics and diet composition on cecal bacteria and growth of chicks. Antibiotics Chemother., *2:* 42.

SAUBERLICH, H. E. 1952 Effect of aureomycin and penicillin upon the vitamin requirement of the rat. J. Nutrition, *46:* 99.

SEVAG, M. G., AND E. L. ROSANOFF 1952 Mechanism of the development of resistance to streptomycin. I. Origin of resistant strains. J. Bact., *63:* 243.

SIEBURTH, J. M., J. GUTIERREZ, J. MCGINNIS, J. R. STERN AND B. H. SCHNEIDER 1951 Effect of antibiotics on intestinal microflora and on growth of turkeys and pigs. Proc. Soc. Exp. Biol. Med., *76:* 15.

SIEBURTH, J. M., J. MCGINNIS AND C. E. SKINNER 1952 The effect of terramycin on the antagonism of certain bacteria against species of proteus. J. Bact., *64:* 163.

STARR, M. P., AND D. M. REYNOLDS 1951 Streptomycin resistance of coliform bacteria from turkeys fed streptomycin. Bact. Proc., 15.

STOKSTAD, E. L. R., AND T. H. JUKES 1951 Effect of various levels of vitamin B_{12} upon growth response produced by aureomycin in chicks. Proc. Soc. Exp. Biol. Med., *76:* 73.

VIJAYARAGHAVAN, P. K., E. A. MURPHY AND M. S. DUNN 1952 The effect of aureomycin on the growth of mice. Arch. Biochem. Biophys., *36:* 127.

WAISMAN, H. A., M. GREEN, J. CRAVIOTO-MUNOZ, A. RAMENCHIK AND J. B. RICHMOND 1951 Role of aureomycin and citrovorum factor in "folic acid" deficiencies. Proc. Soc. Exp. Biol. Med., *76:* 384.

WHITEHILL, A. R., J. J. OLESON AND B. L. HUTCHINGS 1950 Stimulatory effect of aureomycin on the growth of chicks. Ibid., *74:* 11.

WILLIAMS, W. L., R. R. TAYLOR, E. L. R. STOKSTAD AND T. H. JUKES 1951 Mechanism of the growth-promoting effect of aureomycin in chicks. Fed. Proc., *10:* 270.

COMPARISON OF FOUR FEEDING METHODS FOR ASSESSING THE RELATIVE GROWTH-PROMOTING PROPERTIES OF PROTEINS [1]

F. W. SHERWOOD AND VIRGINIA WELDON
Animal Nutrition Section, Animal Industry Department, North Carolina Agricultural Experiment Station, Raleigh

(Received for publication August 1, 1952)

The need for rating different samples of cowpeas in respect to the nutritive quality of their proteins focused attention on methodology. The detection of differences in growth-promoting value rather than the determination of more quantitative expressions of quality was considered to be sufficient for the purpose. Since differences in growth were expected to be small, a sensitive method for detecting these differences was desired.

Both ad libitum and paired feeding (Mitchell and Beadles, '30) have been widely used for comparing the growth-promoting properties of proteins as well as for determining protein efficiencies, as indices of quality (Osborne, Mendel and Ferry, '19). Each method has been criticized, chiefly in respect to its suitability for determining characteristic indices of nutritive value (Boas Fixsen, '34–'35; Palmer and Kennedy, '31; Mitchell, '44; Brody, '45; Barnes and Bosshardt, '46). Both procedures have been found to place proteins in essentially the same rank order of growth-promoting value (Jones and Widness, '46; Murray, '48; Richardson, '48). Hegsted and Worcester ('47) have shown that comparison between proteins may

[1] Published with the permission of the Director of the North Carolina Agricultural Experiment Station as paper 435 of the Journal Series.

be made in terms of gains in body weight instead of the conventional gains per gram of protein eaten.

No direct comparisons of the sensitivity of the two feeding methods for detecting differences in the nutritive value of proteins have been found in the literature. Hamilton and Mitchell ('35) claimed that paired feeding was more precise than the ad libitum procedure usually used in vitamin assays. Mitchell ('44) stated that ad libitum feeding exaggerates large differences and obscures small differences in protein quality. Stewart, Hensley and Peters ('43) and Harte, Travers and Sarich ('47) observed less individual variation within diet groups when a moderate restriction in feed intake was imposed than when the diets were fed ad libitum.

The work reported here was undertaken to obtain information on the relative sensitivities of the ad libitum and the paired feeding techniques when used for comparing the growth-promoting value of cowpea protein with that of casein. At the same time, two additional feeding procedures were included in this study. The details of these additional procedures, the constant protein method and the adjusted protein method, have not been described in the literature.

EXPERIMENTAL

Feeding methods

A small excess of feed was kept before the ad libitum-fed rats at all times. In this method the level of protein was fixed at 12% of the diet, but there was no restriction on the amount of feed consumed. In the paired feeding method the amount of feed given the rat with the better appetite was equal in weight to that eaten by its ad libitum-fed pair mate in the preceding 24 hours. There was no restriction imposed upon the feed intake between replicate pairs.

In the constant protein procedure each animal was given daily 4 gm of the protein diet (containing 0.5 gm protein) mixed with as much of an energy supplement as would be eaten in 24 hours. Thus, the protein intake of all rats, both

within and between the diet groups, was equalized at a constant suboptimum level. This fixed amount of diet furnished adequate minerals and vitamins but did not supply enough energy to meet the needs of the rats; therefore, an additional source of energy was supplied ad libitum. The procedure employed in the adjusted protein method was similar to that in the constant protein method except that when a rat reached 55 gm in body weight, the daily allowance of protein diet was increased from 4.0 to 4.8 gm. When a rat reached a weight of 65 gm, its allowance was increased to 5.6 gm of diet, and so on in steps of 0.8 gm diet (0.1 gm protein) for each 10 gm increase in weight. This adjustment in the amount of feed offered the rats was made so as to allow, in some measure, for the larger requirements as the animal increased in weight. The protein intake by this method varied both within and between the diet groups in proportion to the attained weight of the rats.

Records of feed eaten by the rats subjected to the 4 procedures were made daily over a 4-week feeding period. The rats used in the adjusted protein method were weighed daily; all of the other rats were weighed twice a week.

Diets

All of the diets (table 1) were approximately equal in respect to their content of fat, of minerals, of fiber and of energy. The difference between the gain in body weights of the rats consuming the cowpea diet, no. 1, and those receiving the casein diet, no. 2, was measured by both the ad libitum and the paired feeding techniques. Diets 3 and 4 were employed when the constant protein and the adjusted protein methods were used.

Selection and assignment of rats

Wistar strain weanling rats weighing 40 to 45 gm were kept on a stock diet until they weighed about 50 gm. At this stage, groups of 8 rats of the same sex and as uniform as possible in respect to age, weight and rate of gain since weaning, were selected. The two most nearly identical animals in each group

(replication) were randomly assigned to two adjacent cages in a bank of 8 contiguous, screen-bottom cages. These rats were used for the paired feeding method. The remaining 6 rats were placed at random in the 6 other cages. The other diet-feeding method combinations were randomly assigned to these 6 rats. Ten replications, 5 of males and 5 of females, were used.

TABLE 1

Percentage composition of diets

INGREDIENT	DIET NUMBER				ENERGY SUPPLEMENT
	1[1]	2[1]	3[1]	4[1]	
Cowpeas[2]	62.5	...	65.0	...	...
Casein, purified	...	12.0	...	12.5	...
Cellulose[3]	0.2	3.0	0.1	3.0	...
Mineral mixture[4]	...	4.0	...	4.0	...
Calcium carbonate	1.0	...	1.0	...	...
Sodium chloride	0.5	...	0.5	...	...
Hydrogenated vegetable fat	9.0	10.0	9.0	10.0	10.0
Cod liver oil	1.0	1.0	1.0	1.0	...
Cornstarch	25.8	70.0	23.4	69.5	90.0

[1] One hundred grams of each diet were supplemented with 200 mg choline chloride, 40 mg *p*-aminobenzoic acid, 1 mg nicotinic acid, and 0.40 mg each of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride and calcium pantothenate.

[2] Dried mature "blackeye" cowpeas containing 19.2% crude protein (N × 6.25).

[3] A commercial purified rice bran.

[4] Wesson ('32).

RESULTS AND DISCUSSION

Comparison of methods, based on gains in body weight

No significant effect attributable to sex was indicated by a statistical analysis of the individual data from each of the feeding methods; consequently, the results from both sexes were combined for the ensuing discussion.

Mean feed consumption and mean gain in body weight for the 10 rats on each of the 8 treatments (diet and feeding method combinations) are presented in table 2.

In order to follow the prescribed directions for paired feeding, the basic randomized block design of the experiment was modified by using a matched pair of rats from each replication

for the paired feeding method. Theoretically this modification should result in a smaller experimental error for the paired feeding method. In the other three procedures the random assignment spread the inherent variability of the rats throughout all treatment groups without bias. The difference between the standard deviations of the three methods, other than paired feeding, more nearly reflects the effect of the technique on the experimental error than would have been the case if each method had been tested independently. In spite of the

TABLE 2

Mean feed consumption and gain in body weight of rats subjected to the 4 feeding methods for 28 days

FEEDING METHOD	DIET	SOURCE OF PROTEIN	FEED EATEN	PROTEIN EATEN	WEIGHT GAINS
	no.		gm	gm	gm
Ad libitum	1	Cowpeas	182	21.9	33.4
	2	Casein	236	28.3	69.3
Paired feeding	1	Cowpeas	190	22.8	36.1
	2	Casein	190	22.8	48.9
Constant protein	3	Cowpeas	165 [1]	14.0	20.8
	4	Casein	194 [1]	14.0	36.1
Adjusted protein	3	Cowpeas	169 [1]	16.5	24.7
	4	Casein	218 [1]	20.1	57.7

[1] Basal diet plus energy supplement.

bias in favor of the paired feeding method, its standard deviation (4.10) was nearly the same as that of the constant protein method (4.29). Both of these procedures, in which the protein intake of the rats on one or both of the sources of protein was restricted, had smaller standard deviations than the other two methods in which the rats were more liberally fed. The standard deviations of the ad libitum and the adjusted protein methods were 7.22 and 7.29, respectively.

However desirable a small experimental error may be, if a technique that leads to a small error term also results in a proportionately smaller difference between treatment groups, it is a less sensitive tool for detecting differences. The most sensitive method is the one that gives the greatest ratio of the

difference between two treatment groups to the standard deviation of this difference. The last line of table 3 shows that, by this criterion, the ad libitum method was the most sensitive and the paired feeding method the least sensitive of the 4 methods studied.

The most sensitive procedure for detecting a difference between the responses of two treatment groups may not always be the most desirable for detecting a difference in quality. The greater sensitivity of the ad libitum and the adjusted protein methods was associated with a larger ingestion of casein than

TABLE 3

Sensitivity of the 4 feeding methods as measured by the ratio of the difference in gain of rats receiving the two sources of protein to the standard deviation of this difference

	FEEDING METHOD			
	Ad libitum	Paired	Constant protein	Adjusted protein
Mean difference [1]	35.9	12.8	15.3	33.0
Standard deviation of difference	3.2	1.8	1.9	3.4
Ratio [2]	11.1	7.0	7.9	9.8

[1] Mean gain in body weight of the 10 rats receiving the casein diet minus the mean gain in body weight of the 10 rats receiving the cowpea diet.

[2] Mean difference divided by the standard deviation of the difference. This is the observed "t" value of the method. The "t" value for significance at P = 0.01 is 3.55 (Snedecor, '46).

of cowpea proteins. This unequal protein intake was advantageous if the greater consumption of casein was due to its superior nutritive qualities. If, however, the inequality was mediated by differences in palatability of the two diets, the adjusted protein method more nearly reflected differences in quality, because the protein intake was governed by the response of the rat and not by the flavor, odor, texture or consistency of the diet. The fact that both the ad libitum and the adjusted protein methods gave so nearly the same comparative results indicates that a difference in palatability between the cowpea and the purified casein diets was not a major factor.

Comparison of methods based on protein efficiencies

The objective of the comparison of proteins by any of the methods under discussion was to obtain some information about the nutritive quality of the test protein relative to that of the standard of comparison. The meaning of the term "quality of protein" is somewhat indefinite, since it attempts to express a complex interrelationship between the available amino acids, both singly and collectively, and the requirements of the rat. The weight gains of rats are dependent not only on the quality of the protein but also on the amount eaten per unit of time, the relation of this amount to the needs of the animal for maintenance and for growth, and the relation of this amount of protein to the other nutrients in the diet. These relationships may change as the rat increases in body weight. The formulation of diets and the method of feeding may control one or more of these factors, but no technique has been devised that controls all simultaneously.

The common practice of expressing results as gain in body weight per gram of protein eaten attempts to account for the amount of protein eaten, one of the several factors that affect weight gains. Because of this additional control, protein efficiency does not necessarily measure the same characteristic of a protein as weight gains alone, although the two metameters are closely related. In view of this consideration, protein efficiencies have been computed from the data and used as a basis for comparing the relative sensitivities of the 4 feeding methods. A summary of these computations is given in table 4.

In spite of the larger standard deviation, the adjusted protein method was the most sensitive of the 4 for detecting differences in protein efficiencies. The ad libitum method was slightly less sensitive than the paired feeding and the constant protein techniques. Each ratio (measure of sensitivity) is subject to sampling errors, and the 4 were so nearly alike that there is no clear-cut evidence for the superiority or inferiority of any of the 4 feeding methods when protein efficiencies are used as the metameter.

The sensitivity of the constant protein method was practically the same whether based on gains in body weight or on protein efficiencies. This indicates that there was no advantage in accounting for the amount of protein eaten when this factor was controlled by the experimental conditions. Even in the paired feeding and the adjusted protein methods, with their less rigorous control of protein intake, the sensitivities were much alike whether based on body weight gains or on gains per gram of protein eaten. Only with the ad libitum method, in which the experimental control was limited to the level of protein in the diet, did the sensitivity vary to any extent with

TABLE 4

Sensitivity of the 4 feeding methods as measured by the ratio of the difference in protein efficiencies to the standard deviation of this difference

	FEEDING METHOD			
	Ad libitum	Paired	Constant protein	Adjusted protein
Mean difference [1]	0.93	0.56	1.09	1.40
Standard deviation of difference	0.125	0.074	0.137	0.150
Ratio [2]	7.4	7.6	8.1	9.4

[1] Mean protein efficiency of rats receiving casein diet minus mean protein efficiency of rats receiving cowpea diet.

[2] Mean difference divided by the standard deviation of the difference. These "t" values are greater than 3.55, the "t" required for significance at P = 0.01.

the metameter used. The sensitivity of the ad libitum method when based on gains in weight was the greatest and, when based on protein efficiencies, the least of the 4 feeding methods. There is not enough to be gained by the use of protein efficiencies in any of the feeding methods to compensate for the additional time and labor required for their computation.

The ad libitum method imposes the least experimental control of the 4 methods over the various factors which affect the gain response of the rats. Results from this method are overall comparisons, including protein quality together with extraneous factors, such as palatability, which differentially affect the feed intake of the rats receiving the two proteins

that are compared. When such an over-all comparison suffices for the objective at hand, the ad libitum method is the one of choice because of its sensitivity and because of the simplicity of carrying it out in the laboratory.

The adjusted protein method was almost as sensitive as the ad libitum method but required considerably more of the feeder's time and labor. It is relatively independent of the palatability of the diets and is the preferable method when it is desired to eliminate differences in palatability from the comparison between proteins.

SUMMARY

Four methods of feeding rats have been compared in respect to their relative merits in detecting differences in the growth-promoting properties of casein and cowpea proteins. The methods were: (a) ad libitum feeding, (b) paired feeding, (c) constant protein feeding, and (d) adjusted protein feeding. The latter two methods have been described in detail.

When gain in body weight in a 4-week feeding period was used as the criterion of response, it was found that greater differences in response to the two sources of protein were obtained by the ad libitum and the adjusted protein procedures than from the paired feeding and the constant protein methods. The former two methods also had the greater standard deviations. The methods ranked, in descending order of their sensitivities as measured by the ratio of the difference to the standard deviation of the difference: ad libitum, adjusted protein, constant protein and paired feeding.

There was no advantage in using protein efficiencies in place of gain in body weight as the metameter.

ACKNOWLEDGMENT

The authors wish to thank Dr. H. L. Lucas of the Department of Experimental Statistics for his aid and advice in the statistical treatment of the data.

LITERATURE CITED

BARNES, R. H., AND D. K. BOSSHARDT 1946 The evaluation of protein quality in the normal animal. Ann. N. Y. Acad. Sci., *XLVII:* 273–296.

BOAS FIXSEN, MARGARET A. 1934–35 The biological value of protein in nutrition. Nutrition Abstr. and Rev., *4:* 447–459.

BRODY, S. 1945 Bioenergetics and Growth. Reinhold Publishing Corp., New York, N. Y., p. 1023.

HAMILTON, T. S., AND H. H. MITCHELL 1935 A modification of the Sherman method of studying the multiple nature of vitamins, with an application to vitamin G. J. Nutrition, *10:* 117–128.

HARTE, R. A., J. J. TRAVERS AND P. SARICH 1947 Protein assay by rat growth: A comparison of (A) litter mates vs. randomly selected males and (B) moderate restriction in food intake vs. ad libitum feeding. Ibid., *34:* 363–372.

HEGSTED, D. M., AND JANE WORCESTER 1947 A study of the relation between protein efficiencies and gains in weight on diets of constant protein content. Ibid., *33:* 685–702.

JONES, D. B., AND KATHERINE D. WIDNESS 1946 The comparative growth-promoting value of proteins of wheat germ, corn germ and some other foods of plant and animal origin. Ibid., *31:* 675–683.

MITCHELL, H. H. 1944 Determination of the nutritive value of proteins of food products. Ind. Eng. Chem., Anal. Ed., *16:* 696–700.

MITCHELL, H. H., AND JESSIE R. BEADLES 1930 The paired feeding method in nutrition experiments and its application to the problem of cystine deficiencies in food protein. J. Nutrition, *2:* 225–243.

MURRAY, HAZEL C. 1948 The biological value of the protein of field pea products with a comparison of several methods used for this determination. Ibid., *35:* 257–268.

OSBORNE, T. B., L. B. MENDEL AND EDNA L. FERRY 1919 A method of expressing numerically the growth-promoting value of proteins. J. Biol. Chem., *37:* 223–229.

PALMER, L. S., AND CORNELIA KENNEDY 1931 The fundamental food requirement for the growth of the rat. VI. The influence of the food consumption and the efficiency quotient of the animal. Ibid., *90:* 545–564.

RICHARDSON, C. R. 1948 Southern peas and other legume seeds as a source of protein for the growth of rats. J. Nutrition, *36:* 451–462.

SNEDECOR, G. W. 1946 Statistical Methods. Iowa State College Press, Ames, Iowa, 4th ed.

STEWART, R. A., G. W. HENSLEY AND F. H. PETERS, JR. 1943 Nutritive value of protein. I. The effect of processing on oat protein. J. Nutrition, *26:* 519–526.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339.

GROWTH RESPONSE OF THE RAT TO HETEROLOGOUS PROTEIN ADMINISTERED INTRAPERITONEALLY

ARLENE P. MARTIN, CHARLES R. SHAW AND HAROLD H. WILLIAMS
School of Nutrition and Department of Biochemistry and Nutrition, Cornell University, Ithaca, New York

ONE FIGURE

(Received for publication July 10, 1952)

It appears to be well established that the parenteral administration of certain homologous proteins such as the plasma proteins and hemoglobin will maintain nitrogen equilibrium and replace blood proteins in depleted animals, at least for short periods of time (Whipple, '42; Whipple and Madden, '44; Elman, '47; Meyer et al., '47). Although the parenteral pathway of administration shows little difference with respect to the above criteria of utilization, it has been shown (Pommerenke et al., '35; Terry et al., '48) that plasma administered intraperitoneally is absorbed rapidly and, in some instances, is less disturbing to the animal than when given by vein.

In contrast to the studies involving parenteral administration of homologous proteins, and despite an earlier study by Taylor et al. ('30) indicating that heterologous hemoglobin could be administered successfully, later attempts to employ other heterologous proteins parenterally seem to have been unsuccessful.

Thus Pommerenke et al. ('35) found that protein-fasting dogs could not be maintained in nitrogen equilibrium when horse plasma was administered intravenously or intraperitoneally as the only source of protein.

Fatal effects suggesting the presence of a foreign protein were noted in dogs after intravenous administration of whole bovine serum and plasma (Davis and Eaton, '42). Similar studies by Wangensteen et al. ('40) and Kremen et al. ('42) with human subjects resulted in a negative nitrogen balance.

In the present long-term study it has been demonstrated that raw egg albumen or bovine plasma supplying the only source of protein can be utilized by the young rat for growth when administered intraperitoneally. In contrast to previous studies with heterologous protein administration, the animals exhibited no symptoms of intoxication and appeared to be in an excellent state of health.

PROCEDURE

Twenty-five male albino rats of the Sprague-Dawley strain, averaging 126 $\pm$ 12.6 gm initial weight, were used in a growth experiment lasting for 12 weeks. The animals were divided by weight into 5 equal groups and placed in individual wire cages in an air-conditioned room. They were allowed feed and water ad libitum. Individual feed consumption and animal weights were recorded every two days. Once a week all the animals were given oral supplements of vitamins A and D.[1]

Four groups of animals were fed a non-protein basal diet[2] consisting of the following parts: dextrin 53.3, dextrose 13.3, water-soluble milk vitamin concentrate[3] 10, corn oil[4] 10, rice polish concentrate[5] 6.7 and salt mixture[6] 6.7. The 5th group was fed ground dog chow.[7]

Of the 4 groups of rats receiving the non-protein basal diet, two were injected intraperitoneally with a heterologous pro-

[1] Vitamins A and D were supplied in the form of Mead Johnson's Oleum Percomorphum.

[2] The non-protein basal diet contained 0.319 mg nitrogen per gram as fed.

[3] Borden's Labco XXX water-soluble milk vitamin concentrate.

[4] Mazola.

[5] Borden's Labco rice polish concentrate.

[6] U.S.P. no. 2 salt mixture.

[7] Purina dog chow.

tein once a day, one group being injected with raw egg albumen, the other group with a bovine plasma solution. The remaining two groups, serving as their controls, were supplied the proteins orally by mixing with the basal diet. To be sure that all the protein was eaten, and not separately from the diet, it was mixed with about 8 gm of the basal diet and placed on top of the remaining food in the food containers. In this way, the animals ate the allotted amount of protein each day. These heterologous proteins were the only sources of nitrogen supplied to the animals except for the small amount in the basal diet.[8]

The egg albumen was obtained by cracking fresh eggs, separating the albumen from the yolk, and straining the albumen through cheesecloth. It was necessary to cook the egg albumen for the group of rats receiving it orally in order to inactivate avadin. The bovine plasma was prepared from freshly citrated bovine blood. The plasma was separated by centrifugation, frozen, and dried from the frozen state in a Desivac. After being ground in a micro Wiley mill, the dried plasma was stored at room temperature. A weighed amount of this plasma was mixed with a small portion of the basal diet and fed orally, as described previously, or dissolved in distilled water and injected.

The animals were given 0.86 gm of egg albumen protein or 0.84 gm of bovine plasma protein daily for the duration of the experiment. This was equivalent to the injection of 8 ml of raw egg albumen or 7 ml of bovine plasma solution.

Nitrogen balances, each of 6 to 7 days' duration, were determined during the second, 4th, and 6th weeks of the experiment. The urine was tested, qualitatively, for the presence of protein.

RESULTS

The 12-week average growth rates of the 5 groups of rats are shown in figure 1. After the first week the animals gained a relatively constant amount in weight each week. Group A

[8] See footnote 2, page 164.

serving as stock controls grew at the fastest rate. Animals ingesting egg albumen (group B) gained weight at a faster rate than those being injected (group C). The greater gain in weight by group B may be attributed to the difference in availability of the protein due to cooking the egg or to the loss of some of the injected protein in the urine of group C, or possibly to both causes. There was no significant

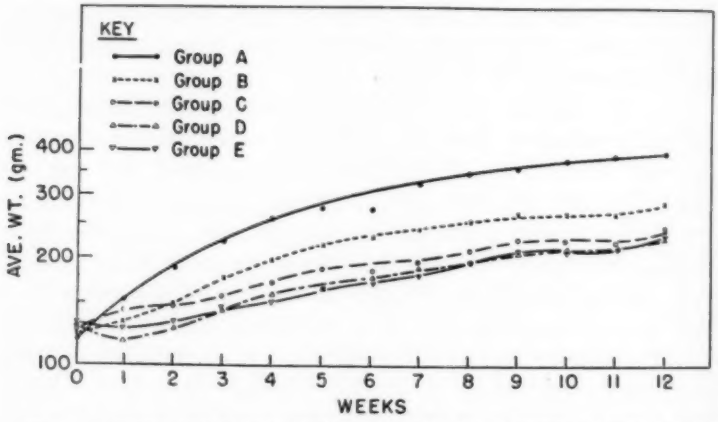


Fig. 1 Average weight gain during 12 weeks for 5 groups of rats fed different sources of protein.

Group A — fed ground dog chow.
Group B — fed non-protein basal diet + cooked egg albumen (oral).
Group C — fed non-protein basal diet + raw egg albumen (injected).
Group D — fed non-protein basal diet + dried bovine plasma (oral).
Group E — fed non-protein basal diet + solution of bovine plasma (injected).

difference in the growth rates of the animals given bovine plasma by the oral (group D) or the intraperitoneal (group E) route.

During the first two weeks, animals of group C were more noticeably distended than were those of group E; following this period, however, none of the injected animals appeared distended. Rats of groups D and E were the only animals to lose weight during the first week.

The non-protein basal diet fed during the 10th and 11th week lacked the usual portion of rice bran.[9] The animals ate less food and did not gain their usual amounts of weight during this time.

TABLE 1

Mean initial and final weights of 5 groups of rats that received protein orally or by intraperitoneal injection

GROUP[1]	INITIAL WT.	FINAL WT.	WT. GAIN
	gm	gm	gm
	Mean S.D.[2]	Mean S.D.	Mean S.D.
A	119 ± 23	392 ± 17	273 ± 29
B	122 ± 20	285 ± 29	163 ± 21
C	127 ± 13	224 ± 20	118 ± 29
D	122[3] ± 10	227 ± 13	105 ± 22
E	127[4] ± 11	228 ± 31	101 ± 32

[1] Group A — ground dog chow.
Group B — non-protein basal diet + cooked egg albumen (oral).
Group C — non-protein basal diet + raw egg albumen (injected).
Group D — non-protein basal diet + dried bovine plasma (oral).
Group E — non-protein basal diet + solution bovine plasma (injected).

[2] S.D. — standard deviation.

[3] Mean and S.D. calculated for three animals. The remaining two animals grew steadily for 8 and 10 weeks. The former was sacrificed after gaining 58 gm and then catching his teeth on the wire cage. The latter animal died after gaining 132 gm, losing 67 gm in the next two weeks. The cause of death was undetermined.

[4] Mean and S.D. calculated for 4 animals. This animal died the 10th week after having gained 71 gm. The cause of death was undetermined.

The weight changes after 12 weeks for each group are summarized in table 1. Nearly all of the animals fed the non-protein basal diet gained over 100 gm in weight during the 12 weeks.

Animals injected daily with raw egg albumen averaged 9.8 gm weekly gain in weight, whereas those receiving it orally averaged 13.6 gm. Animals injected with the bovine plasma solution gained an average of 8.4 gm a week compared to 8.8 gm for those receiving the plasma orally.

[9] See footnote 5, page 164.

The animals ate between 11.7 and 14.8 gm daily of the non-protein basal diet, groups B and C consuming slightly more food than groups D or E. Both groups of controls ate their protein ravenously.

Nitrogen balance studies demonstrated that all animals on which determinations were made were in positive nitrogen balance, retaining from 17 to 41% of their nitrogen intake.

Qualitative urinary protein tests for albumen with salicylic acid and acetic acid showed that only the urine from those rats injected with raw egg albumen gave a positive test, thereby indicating a loss of protein. This is in agreement with the findings of others and can perhaps be best explained by the extremely small molecular size of the egg albumen molecule.

DISCUSSION

In an earlier study by Holman et al. ('34) whole dog plasma was administered to protein-depleted dogs. Oral administration failed to maintain positive nitrogen balance, in contrast to a parallel experiment in which the same amount of plasma was injected intravenously. From these observations the authors concluded that the injected plasma protein was more completely utilized. This interpretation indicated that parenterally administered plasma must be metabolized in a manner dissimilar to its metabolism following oral ingestion.

Howland and Hawkins ('38) phlorhizinized dogs in order to study the metabolism of plasma administered by both routes. As was to be expected, following oral administration an excess amount of nitrogen and sugar was eliminated in the urine. The breakdown of intravenously injected protein was apparently different, however, since they were unable to find excess nitrogen or sugar being eliminated.

It is evidence of this nature that has led Madden and Whipple ('40) to postulate a theory of protein catabolism in which the protein, when injected, is broken down into relatively large aggregates of amino acids which can then be reassembled by the cells. This takes account of a minimum alteration in the structure of the protein molecule, whereas

maximum change occurs when the protein is ingested. More recent data favoring this view are presented by McKee and Stewart ('50), McKee et al. ('50, '52) and Yuile et al. ('51). Others, Eckhardt et al. ('48) and the studies reviewed by Elman ('47), have presented evidence supporting theories that differ from that of Whipple and his associates.

In the present study evidence has been presented which indicates that the animals receiving the bovine plasma solution intraperitoneally were able to utilize this protein for growth as well as their controls which received the plasma orally. It is difficult to carry out the same comparison with those animals receiving the egg albumen, as the injected animals lost an unknown quantity in the urine. The animals receiving the egg albumen orally did grow at a much faster rate than the injected animals. It should not be concluded, however, that this is in apparent disagreement with the above observations. The choice of egg albumen does not aid in answering the question of degree of utilization, for not only was it necessary to cook the albumen before oral feeding, but also, as mentioned above, some of the injected protein was lost in the urine, thereby making a difference in the amount of protein available to the animal.

It is of considerable interest, nevertheless, that the rat can utilize a foreign protein for growth. Although even homologous proteins can prove occasionally to be fatal when administered parenterally to dogs, as demonstrated by Daft et al. ('38), and by Miller et al. ('45), and as reviewed by Elman ('47), it is rather surprising that the two heterologous proteins used in this experiment should not have proved toxic. Evidently the rat not only is more resistant to these foreign bodies, but also it can utilize these foreign proteins apparently with equal efficiency by the oral or intraperitoneal route.

SUMMARY

Evidence has been presented which indicates that the heterologous intact proteins, raw egg albumen or a solution of

bovine plasma, administered intraperitoneally can be utilized by young rats for growth over a long period of time.

Positive nitrogen balances were obtained regardless of whether the proteins were given orally or intraperitoneally.

No signs of intoxication were noted following the injection of these proteins and the injected animals were as healthy and active as their controls which were given the protein orally.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Miss Martha A. Simons for her technical assistance with parts of this study.

LITERATURE CITED

DAFT, F. S., F. S. ROBSCHEIT-ROBBINS AND G. H. WHIPPLE 1938 Plasma protein given by vein and its influence upon body metabolism. J. Biol. Chem., *123:* 87–98.

DAVIS, H. A., AND A. G. EATON 1942 Comparative effects of horse serum, horse serum albumin and horse serum globulin in experimental shock. Proc. Soc. Exp. Biol. Med., *49:* 359–361.

ECKHARDT, R. D., J. H. LEWIS, T. L. MURPHY, W. H. BATCHELOR AND C. S. DAVIDSON 1948 Chemical, clinical and immunological studies on the products of human plasma fractionation. XXXIV. Comparative studies on the nutritive value of orally and intravenously administered human serum albumin in man. J. Clin. Invest., *27:* 119–134.

ELMAN, R. 1947 The use of protein and protein hydrolysates for intravenous alimentation. Advances in Protein Chem., *3:* 269–293.

HOLMAN, R. L., E. B. MAHONEY AND G. H. WHIPPLE 1934 Blood plasma protein given by vein utilized in body metabolism. II. A dynamic equilibrium between plasma and tissue proteins. J. Exp. Med., *59:* 269–282.

HOWLAND, J. W., AND W. B. HAWKINS 1938 Protein metabolism, protein interchange and utilization in phlorhizinized dogs. J. Biol. Chem., *123:* 99–110.

KREMAN, A. J., H. HALL, H. K. KOSCHNITZKE, B. STEVENS AND O. W. WANGENSTEEN 1942 Studies on the intravenous administration of whole bovine plasma and serum to man. Surgery, *11:* 333–355.

MADDEN, S. C., AND G. H. WHIPPLE 1940 Plasma proteins: their source, production and utilization. Physiol. Rev., *20:* 194–217.

MCKEE, F. W., AND W. B. STEWART 1950 Passage of radioactive erythrocytes from the peritoneal cavity into the blood stream during experimental ascites. J. Exp. Med., *91:* 599–605.

McKee, F. W., W. G. Wilt, Jr., R. E. Hyatt and G. H. Whipple 1950 The circulation of ascitic fluid. Interchange of plasma and ascitic fluid protein as studied by means of C^{14}-labeled lysine in dogs with constriction of the vena cava. Ibid., *91:* 115–122.

McKee, F. W., C. L. Yuile, B. G. Lamson and G. H. Whipple 1952 Albumin and globulin circulation in experimental ascities. Relative rates of interchange between plasma and ascitic fluid studied with C^{14}-labeled proteins. Ibid., *95:* 161–172.

Meyer, F. L., J. W. Hirshfeld, W. E. Abbott, M. A. Pilling, H. H. Williams and A. J. Richards 1947 Nitrogen balance and blood volume studies in man during and following repeated plasma transfusion. Am. J. Med. Sci., *213:* 160–169.

Miller, L. L., F. S. Robscheit-Robbins and G. H. Whipple 1945 Hemoglobin and plasma protein. Their relation to internal body protein metabolism. J. Exp. Med., *81:* 405–422.

Pommerenke, W. T., H. B. Slavin, D. H. Kariher and G. H. Whipple 1935 Dog plasma protein given by vein utilized in body metabolism of dog. Horse plasma and dog hemoglobin not similarly utilized. Ibid., *61:* 283–297.

Taylor, G. B., E. J. Manwell, F. S. Robscheit-Robbins and G. H. Whipple 1930 Blood regeneration in severe anemia. XX. Conservation of sheep and goose hemoglobin given intravenously to form dog hemoglobin. Am. J. Physiol., *92:* 408–413.

Terry, R., W. E. Sandrock, R. E. Nye, Jr., and G. H. Whipple 1948 Parenteral plasma protein maintains nitrogen equilibrium over long periods. J. Exp. Med., *87:* 547–559.

Wangensteen, O. W., H. Hall, A. Kremen and B. Stevens 1940 Intravenous administration of bovine and human plasma to man: proof of utilization. Proc. Soc. Exp. Biol. Med., *43:* 616–621.

Whipple, G. H. 1942 Hemoglobin and plasma proteins: their production, utilization and interrelation. Am. J. Med. Sci., *203:* 477–489.

Whipple, G. H., and S. Madden 1944 Hemoglobin, plasma protein and cell protein: their interchange and construction in emergencies. Medicine, *23:* 215–224.

Yuile, C. L., B. G. Lamson, L. L. Miller and G. H. Whipple 1951 Conversion of plasma protein to tissue protein without evidence of protein breakdown. Results of giving plasma protein labeled with carbon^{14} parenterally to dogs. J. Exp. Med., *93:* 539–557.

COBALT AND VITAMIN B_{12} IN SHEEP

I. DISTRIBUTION OF RADIOCOBALT IN TISSUES AND INGESTA

P. ROTHERY,[1] J. M. BELL[2] AND J. W. T. SPINKS[3]

Departments of Animal Husbandry and Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

(Received for publication August 5, 1952)

Since cobalt was first reported to be a dietary essential for ruminants, investigators in many parts of the world have maintained sheep or cattle on cobalt-deficient rations and demonstrated the beneficial effects of cobalt supplementation. The isolation of vitamin B_{12} (Rickes et al., '48; Smith, '48) and the subsequent discovery that it contained cobalt stimulated new interest in the role played by the element. Most, if not all, non-ruminants require dietary vitamin B_{12}, whereas ruminants apparently derive their requirements of this vitamin through microbial synthesis in the gastrointestinal tract, and particularly in the rumen. Becker et al. ('49) postulated that cobalt deficiency in sheep might be primarily a vitamin B_{12} deficiency, but injections of the vitamin failed to effect a response. Later studies in which larger doses of the vitamin were used did, however, result in improvement (Smith et al., '51). Obviously the nutrients cobalt and vitamin B_{12} are related but the vitamin B_{12} therapeutic dose appears large in relation to the dietary cobalt requirements as cited by Maynard ('51). In addition, sheep consuming diets adequate in cobalt (Hale et al., '50) were shown to possess about 50 μg vitamin B_{12} per 100 gm dry rumen ingesta, an amount insufficient to account for more than

[1] Formerly graduate student, Department of Animal Husbandry.

[2] Associate Professor of Animal Husbandry.

[3] Professor of Chemistry.

about 20% of the ingested cobalt, disregarding possible rumen absorption.

Several reports have dealt with the distribution of radiocobalt in ruminant tissues (Comar et al., '46a, b, '47; Keener et al., '51), with samples usually taken soon after a few doses of active material were given. There is need to gather further data on ruminants receiving near-normal levels of dietary cobalt (labelled) over a period of time, even though the works of Cuthbertson et al. ('50) and Copp and Greenberg ('41) indicate little difference between tissue "activity" levels resulting from a few heavy doses as compared to smaller doses administered over prolonged periods (in rats). Such information would facilitate the gathering of reliable data on the incorporation of radiocobalt in vitamin B_{12} (Abelson and Darby, '49; Rosenblum and Woodbury, '51), and the extent to which vitamin B_{12} is a function of cobalt metabolism in ruminants.

This report concerns the distribution of cobalt in lambs fed low and moderate levels of radioactive cobalt over periods of three to 4 months.

EXPERIMENTAL

In experiment I (1949) 4 healthy range wether lambs weighing about 30 kg each were confined to metabolism cages and fed a ration consisting of lowland native hay (believed low in cobalt on the basis of earlier response to cobalt supplementation), oats, skim milk powder and a "cobalt-free" mineral mixture. Two of the lambs were given 100 μg labelled cobalt (Co^{60}) orally by capsule every other day until they were slaughtered after 123 days. A 10-day balance trial was conducted near the termination of the experiment.

In the following year (experiment II) lambs were secured at or soon after birth and given a diet of reconstituted, vitamin-fortified skim milk. Because of a delay in receiving special ration ingredients, the lambs had access for a few weeks to a mixture of ground oat hulls (0.2 p.p.m. Co), ground yellow corn, and skim milk powder fortified with vitamins A and D and minerals, less cobalt. When about 100 days of age 6 lambs

were allotted to a semi-purified diet of the following percentage composition: cellulose [4] 32.6, cornstarch 35, skim milk powder 20, minerals [5] 7, lard 3, and urea 2.4.

The lambs readily adapted themselves to this diet and there was noticeable improvement in digestive disorders which had occurred and caused some mortality up to this time.

Each of 4 lambs was given 500 μg of labelled cobalt daily by gelatin capsule and two of these received 15 μg vitamin B_{12} intravenously at weekly intervals. Two others received neither treatment over the experimental period of approximately 90 days.

In this study the lambs were confined to individual pens bedded with shavings, except for one day each week when exercise was allowed in an outdoor pen free of vegetation. Feed and water were provided twice daily and records were kept of feed consumption and weight gains.

At the time of slaughter the gastrointestinal contents and various tissues were removed quantitatively and weighed. Tissues were quick-frozen and gastrointestinal contents were rendered slightly acid (HCl), autoclaved in sealers and sealed tightly (Rothery, '51). Later, total activity of Co^{60} and activity of n-butanol extracts were measured with a dipping-type counter [6] immersed in 100 ml of a slurry of tissue or ingesta. Data pertaining to the organically-bound cobalt or vitamin B_{12} will be presented elsewhere following further study.

RESULTS AND DISCUSSION

The lambs in the first experiment fed natural feedstuffs were obviously not greatly dependent for their cobalt on that supplied as a supplement, for there was no change noted in erythrocyte count and no depression in appetite or weight gains when cobalt was not added. However those animals fed

[4] Ground unbleached sulphite wood pulp.

[5] The minerals were: 7.26 kg $CaHPO_4 \cdot 2H_2O$; 7.26 kg $CaCO_3$; 9.49 kg K_2HPO_4; 2 kg $NaH_2PO_4 \cdot 2H_2O$; 4.54 kg $MgSO_4$; 270 gm $FeSO_4 \cdot 7H_2O$; 270 gm $MnSO_4 \cdot 7H_2O$; 4.09 gm KI; 2.72 gm $CuSO_4 \cdot 5H_2O$; 1.36 gm NaF; 3.63 gm $ZnSO_4 \cdot 7H_2O$, and 2.27 kg NaCl.

[6] Radiation Counter Laboratories.

the semi-purified diet, less cobalt, did display gradual loss of appetite and weight as well as some degree of pica. The feeding of 50 (i.e., 100 μg every other day) and 500 μg cobalt per day provided minimum cobalt intakes of about 0.05 and 0.5 p.p.m., respectively, and these levels were associated with marked differences in tissue and ingesta cobalt concentrations (table 1). Despite such differences, there was no indication of tissue deficiency at the low intake level because the lambs receiving an average of 50 μg daily were in cobalt balance according to the urine and feces data of the 10-day balance trial. About 6 to 8% of the ingested cobalt was excreted in the urine.

The tissue cobalt concentrations were well in excess of those reported by Comar and Davis ('47) in cattle fed radiocobalt. The differences may be due to the fact that the cattle received few doses of cobalt and the levels were smaller per unit of body weight than in the case of the lambs given 500 μg cobalt daily. In addition, some time would be required to permit the rumen microorganisms typical of a cobalt-deficient ration to readjust themselves to a cobalt-supplemented medium, and consequently it is not necessarily strange that the results of cobalt metabolism may differ in the two reports.

These data confirm the liver as the organ of highest cobalt concentration, with significant quantities in the heart, kidney and pancreas. The two lambs (IX and X) that received vitamin B_{12} injections had relatively large amounts of cobalt in their spleens, a finding difficult to interpret from this study. There were no important differences in cobalt concentrations in tissues of the gastrointestinal tract. Cobalt was definitely detected in the blood of only one lamb.

Within the digestive tract cobalt concentrations were somewhat variable, as might be expected from the differences in time lapse between last cobalt administration and slaughter, and from the cobalt not being uniformly mixed with the feed as offered. There was undoubtedly a tendency for increased concentration as the ingesta approached the rectum, but this can be largely accounted for by dry matter digestion and decreased moisture content.

TABLE 1

Concentration of labelled cobalt in tissues and ingesta of lambs fed Co^{60} for 90–123 days, as related to the lapse of time since last cobalt administration (μg Co/kg wet tissue)

SAMPLE	HOURS SINCE LAST Co^{60} FED					
	24	24	3	5	24	24
Lamb number	II	IV	VIII	IX	VII	X
Cobalt fed/day, μg	50	50	500	500	500	500
Tissues						
Blood [1]	✳ [2]	✳	..	..	1	..
Muscle [1]	1	2	4	7	3	4
Bile	..	..	0	0	0	0
Brain	..	..	5	17	1	7
Heart	..	..	23	15	1	51
Kidney	✳	✳	7	57	48	80
Liver	3	7	124	171	lost	126
Pancreas	✳	✳	41	33	17	27
Spleen	✳	✳	7	29	0	26
Thyroid	✳	✳	✳	0	✳	0
Rumen	..	..	10	13	8	8
Reticulum	..	..	14	lost	15	15
Omasum	..	..	23	12	19	17
Abomasum	..	..	40	24	22	19
Small intestine	..	..	36	49	22	23
Cecum	..	..	28	47	28	27
Colon	..	..	47	34	45	38
Rectum	..	..	33	20	49	31
Gastrointestinal contents						
Rumen	1	3	147	116	81	31
Reticulum	2	16	208	192	24	29
Omasum	9	17	..	..	..	..
Abomasum	✳	7	109	146	47	43
Small intestine	4	9	116	292	139	17
Cecum	16	25	441	978	73	186
Colon	} 24	} 17	524	1251	434	116
Rectum	}	}	1156	2343	330	1346

[1] Estimated according to the data presented by L. R. Wallace, J. Agr. Sci., *38*: (3), 243–302, 1948.

[2] "✳" indicates trace of activity only.

TABLE 2

Distribution of radiocobalt among the various tissues and gastrointestinal contents of lambs fed Co^{60} for prolonged periods, as related to the lapse of time since the last administration of Co^{60} (percentage of total body and ingesta cobalt)

SAMPLE	HOURS SINCE LAST COBALT FED					
	24	24	3	5	24	24
Lamb number	II	IV	VIII	IX	VII	X
Cobalt fed/day, μg	50	50	500	500	500	500
Tissues						
Blood (est. 5% of live weight)	✳[1]	✳	0	0	0.51	0
Muscle (est. 40% of dressed weight)	35.00	42.64	5.41	6.49	7.47	14.38
Bile	..	..	0	0	0	0
Brain	..	..	0.05	0.20	0.10	0.22
Heart	..	..	0.31	0.16	0.03	1.86
Kidney	✳	✳	0.06	0.48	0.62	1.62
Liver	2.48	2.74	5.08	6.01	(lost)	14.92
Pancreas	✳	✳	0.14	0.08	0.11	0.28
Spleen	✳	✳	0.03	0.09	0	0.39
Thyroid	✳	✳	✳	0	✳	0
Rumen	..	..	0.40	0.41	0.59	0.81
Reticulum	..	..	0.14	(lost)	0.28	0.33
Omasum	..	..	0.10	0.06	0.17	0.19
Abomasum	..	..	0.38	0.20	0.48	0.53
Small intestine	..	..	1.00	1.09	1.34	2.64
Cecum	..	..	0.11	0.14	0.10	0.26
Colon	..	..	0.17	0.11	0.52	0.66
Rectum	..	..	0.41	0.24	0.92	1.29
Total tissue activity (%)	37.48	45.38	13.79	15.76	13.24	40.38
Gastrointestinal tract contents						
Rumen	8.71	19.39	42.89	24.38	41.69	16.88
Reticulum	0.97	2.69	3.47	2.41	0.40	0.72
Omasum	2.44	3.92	..	..	..	..
Abomasum	✳	1.58	3.13	4.43	1.87	1.97
Small intestine	7.49	7.92	2.68	7.10	10.61	1.80
Cecum	31.70	9.77	8.83	17.78	1.49	10.77
Colon	11.22	9.35	11.46	8.10	20.35	4.47
Rectum			13.75	20.07	10.37	23.00
Total ingesta activity (%)	62.53	54.62	86.21	84.27	86.78	59.61

[1] "✳" indicates trace of activity only.

In table 2 the apparent distribution of cobalt for each organ is given as the percentage of total body and ingesta cobalt for both experiments. Muscle thus ranks with liver as a site of cobalt storage in spite of its lower concentration per unit weight. The small intestine contained more cobalt than any other region of the gastrointestinal tract. This may be of special significance in cobalt metabolism, since the weight of fresh intestine tissue was exceeded by that of rumen tissue, and since the average rumen contained 68% of the total ingesta (wet basis).

Of some interest are the low values for omasums in lambs fed 500 μg cobalt daily. It is probable that the soft and highly digestible nature of the cellulose used in their diets resulted in underdevelopment of the omasum. The organ was invariably small and contained little, if any ingesta, whereas the same organs in lambs fed hay contained up to 3% of the ingesta.

In general the results of this study indicate that both tissue and ingesta concentrations of radiocobalt can be increased by increasing the level of dietary cobalt (Co^{60}) and by feeding it regularly over a period of time. The prolonged feeding of labelled cobalt, as contrasted to limited or irregular doses, to sheep receiving low-cobalt rations would also appear to permit better microbial adjustment in the rumen, and a more accurate appraisal of vitamin B_{12}-cobalt interrelationships in tissue and ingesta.

SUMMARY

Data on tissue and ingesta cobalt concentrations are presented for lambs fed natural and semi-purified diets, and receiving, respectively, 50 and 500 μg of labelled cobalt daily over periods of 90 to 123 days.

Cobalt concentrations in the various organs reflected levels of intake and greatly exceeded those reported by Comar and Davis ('47) for cattle fed limited amounts of cobalt. The highest concentrations of cobalt were found in liver, heart, kidney and pancreas, although muscle, by reason of its relatively greater mass, equalled liver as a site of total cobalt

storage. The small intestine tissue had "activity" well in excess of that of other gastrointestinal tissues.

Some possible advantages are presented for the regular and prolonged feeding of radiocobalt as a means of quantitatively assessing the roles of cobalt and vitamin B_{12} in ruminants.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Pacific Mills, Ltd., Vancouver, and to Riordon Sales Corporation, Ltd., Montreal, for the generous supplies of sulphite wood pulp; also to Merck and Company, Inc., Rahway, N. J., for a contribution of crystalline vitamin B_{12}.

Thanks are due Mr. Kenneth Williams and Mr. Robert Steele for assistance in certain phases of the work.

This project was supported by a grant from the National Research Council of Canada, Ottawa.

LITERATURE CITED

ABELSON, P. H., AND H. H. DARBY 1949 The synthesis of vitamin B_{12} in the digestive system of the sheep. Science, *110:* 566.

BECKER, D. E., S. E. SMITH AND J. K. LOOSLI 1949 Vitamin B_{12} and cobalt deficiency in sheep. Ibid., *110:* (2846), 71.

COMAR, C. L., AND G. K. DAVIS 1947 Cobalt metabolism studies. III. Excretion and tissue distribution of radioactive cobalt administered to cattle. Arch. Biochem., *12:* 257.

COMAR, C. L., G. K. DAVIS AND R. F. TAYLOR 1946a Cobalt metabolism studies: Radioactive cobalt procedures with rats and cattle. Ibid., *9:* 149.

COMAR, C. L., G. K. DAVIS, R. F. TAYLOR, C. F. HUFFMAN AND R. E. ELY 1946b Cobalt metabolism studies. II. Partition of radioactive cobalt by a rumen fistula cow. J. Nutrition, *32:* 61.

COPP, D. H., AND D. M. GREENBERG 1941 Studies on mineral metabolism with the aid of artificial radioactive isotopes. VI. Cobalt. Proc. Natl. Acad. Sci., *27:* 153.

CUTHBERTSON, W. F. J., A. A. FREE AND D. M. THORNTON 1950 Distribution of radioactive cobalt in the rat. Brit. J. Nutrition, *4:* 42.

HALE, W. H., A. L. POPE, P. H. PHILLIPS AND R. BOHSTEDT 1950 The effect of cobalt on the synthesis of vitamin B_{12} in the rumen of sheep. J. Animal Sci., *9:* 414.

KEENER, H. A., R. R. BALDWIN AND G. P. PERCIVAL 1951 Cobalt metabolism studies with sheep. Ibid., *10:* 428.

MAYNARD, L. A. 1951 Animal Nutrition. McGraw-Hill Book Co., Inc., N. Y., N. Y.

RICKES, E. L., N. G. BRINK, F. R. KONIUSZY, T. R. WOOD AND K. FOLKERS 1948 Crystalline vitamin B_{12}. Science, *107:* 396.

ROSENBLUM, C., AND D. T. WOODBURY 1951 Cobalt^{60} labelled vitamin B_{12} of high specific activity. Ibid., *113:* 215.

ROTHERY, P. 1951 Some physiological aspects of cobalt and vitamin B_{12} in sheep. I. Distribution of radiocobalt in tissues and ingesta. II. On mouse assay methods for vitamin B_{12}. Master of Science thesis, University of Saskatchewan.

SMITH, E. L. 1948 Purification of anti-pernicious anemia factors from liver. Nature, *161:* 638.

SMITH, S. E., B. A. KOCK AND K. L. TURK 1951 Cobalt deficiency and vitamin B_{12}. J. Nutrition, *44:* 455.

THE METABOLIC FECAL NITROGEN EXCRETION OF THE YOUNG CALF AND THE TRUE DIGESTIBILITY OF CASEIN [1]

G. P. LOFGREEN AND MAX KLEIBER
Department of Animal Husbandry, University of California, Davis

ONE FIGURE

(Received for publication August 4, 1952)

In earlier reports from this institution (Lofgreen, Kleiber and Smith, '51, '52; Lofgreen, Kleiber and Luick, '52) experiments were described in which the objectives were to study the digestion and absorption of the phosphorus organically bound in the casein molecule, the distribution of injected and fed radioactive phosphorus (P^{32}) in the tissues of the calf's body, and the determination of the metabolic fecal phosphorus excretion of the calf under conditions of milk feeding. The present paper reports studies on the extent of the digestion of the nitrogen in casein by young calves and the excretion of metabolic fecal nitrogen under conditions of milk feeding.

Because of the difficulty of getting animals to consume adequate amounts of a nitrogen-free ration, and because of the abnormal conditions to which such a treatment subjects an animal, the metabolic nitrogen excretion of the young calf consuming only milk has been largely neglected. Blaxter and Wood ('51) have recently studied this problem with calves on a liquid diet. The metabolic fecal nitrogen excretion was measured during a period on a nitrogen-free diet. Bosshardt and Barnes ('46) have presented evidence indicating that

[1] This study was partially supported by a grant from the U. S. Atomic Energy Commission.

values for metabolic nitrogen excretion obtained during the feeding of nitrogen-free or nitrogen-low diets may be unreliable when applied to conditions of protein feeding. Any method which could be devised to measure the metabolic nitrogen excretion while the animal is being fed a normal amount of protein would not be subject to the above disadvantages and would provide a means whereby this problem could be studied on young calves, where data are lacking.

Lofgreen, Kleiber and Luick ('52) determined the metabolic fecal phosphorus excretion of the calf on a liquid diet by feeding casein labeled with radioactive phosphorus, P^{32}. The ratio of total phosphorus to P^{32} was determined in the casein and in the feces. The increase in this ratio in the feces was considered to be due to the dilution of the P^{32} by the unlabeled metabolic phosphorus. A similar method may be used for the determination of metabolic fecal nitrogen by use of the ratio of nitrogen to radioactive phosphorus ($N : P^{32}$ ratio) in the casein and in the feces. A theoretical outline of such a method is presented in figure 1. This method is based upon the assumptions that the liberation of phosphorus from casein is proportional to the digestion of the protein, that the portion of the casein which passes through the tract undigested has the same $N : P^{32}$ ratio as that which was fed, and that all the isotope appearing in the feces comes directly from the undigested casein. It has been shown that animal phosphatases will not attack the phosphorus-amino acid linkage to any great extent in the intact casein molecule (Kay, '34). It is also true that during the digestion of casein there is some liberation of certain phosphorus-containing compounds (Kay, '34). These, however, are apparently quickly attacked by phosphatases, since Lofgreen et al. ('51) found that phosphorus was probably absorbed in the inorganic form and that measurable levels of P^{32} were found in the blood stream as early as 15 minutes after the feeding of labeled casein. The feces will contain a small amount of P^{32} which has been absorbed and re-excreted. A correction can be made for this re-excretion according to a method previously described by Lofgreen,

Kleiber and Smith ('52). It appears, therefore, that the assumptions made are reasonable.

EXPERIMENTAL

Casein labeled with P^{32} was prepared and incorporated into purified liquid diets as described in an earlier report (Lofgreen et al., '51). Four calves were fed the purified diet containing non-labeled casein from three days of age until

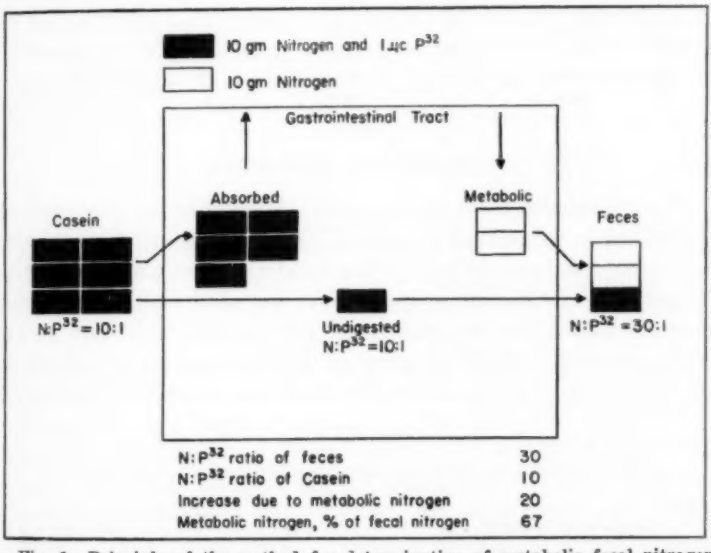


Fig. 1 Principle of the method for determination of metabolic fecal nitrogen by feeding P^{32}-labeled casein.

they were used in these studies. They were then given one or two feedings of the diet containing labeled casein as the sole source of nitrogen. Fecal samples were collected quantitatively for periods of 24 or 36 hours after the initial feeding of the labeled casein. The information concerning the 4 calves used in these studies is presented in table 1.

Either carmine or lampblack was used as a dye to give a rapid visual indication as to when the feces being excreted

corresponded to the feeding of labeled casein. The N:P^{32} ratios of the casein and of the fecal samples containing the dye were determined and used for calculation of the proportion of the fecal nitrogen which was of metabolic origin.

TABLE 1

Description and treatment of calves

CALF NO.	BREED	AGE	WEIGHT	FEEDINGS OF LABELED CASEIN	TOTAL P^{32} ADMINISTERED
		days	*kg*		*microcuries*
1	Jersey	72	59.5	1	9.5
2	Jersey	50	32.3	1	17.1
13	Jersey	25	27.0	2	22.1
14	Holstein	14	36.0	2	29.5

RESULTS AND DISCUSSION

The metabolic fecal nitrogen as determined by the method described is presented in table 2. In the case of three of the calves approximately half of the fecal nitrogen was of metabolic origin. The higher proportion of metabolic nitrogen in the case of calf 1 can be accounted for by the fact that straw had been used as bedding prior to the trial and an examination of the rumen contents after the trial was completed indicated

TABLE 2

The metabolic fecal nitrogen excretion of calves fed labeled casein

CATEGORY OF INTEREST	CALF NO.			
	1	2	13	14
Time of sampling feces, hours after initial feeding	10	18	30	30
N: P^{32} ratio of feces corrected for re-excretion of P^{32} [1]	5,000	667	1,260	1,240
N: P^{32} ratio of casein [1]	1,270	380	602	602
Increase due to metabolic N	3,730	287	658	638
Metabolic N:				
% of fecal N	75	43	52	51
gm per day	2.85	1.05	0.86	1.38
gm per day per kg body weight	0.048	0.033	0.034	0.041
gm per 100 gm dry matter intake	0.34	0.21	0.25	0.29

[1] Milligrams N per μc P^{32}.

that a large amount of straw had been consumed. Any nitrogen present in the straw would be determined as metabolic nitrogen. The consumption of a roughage would also tend to increase the excretion of metabolic nitrogen. Even though the calf consumed significant quantities of straw, it is possible to obtain a valid measure of the true digestibility of the casein nitrogen, since the nitrogen of the straw would be included in the metabolic fraction.

Mitchell ('26) and Schneider ('34, '35) found that metabolic fecal nitrogen excretion was related to the dry matter intake when the level of feeding was at maintenance or above. Blaxter and Mitchell ('48) used a value of 0.45 gm of nitrogen per

TABLE 3

Relation of body weight to metabolic nitrogen excretion

CALF WEIGHT	METABOLIC NITROGEN, EXPRESSED PER 100 GM DRY MATTER INTAKE	REFERENCE
kg	*gm*	
359	0.53	Swanson and Herman ('43)
160	0.49	Harris, Work and Henke ('42)
97	0.37	Harris and Loosli ('44)
39	0.27	The present report

100 gm of dry matter intake for the metabolic nitrogen excretion of Holstein heifers varying in weight from 200 to 1,200 lb. The average of the metabolic nitrogen excretion values presented in table 2 is 0.27 gm of nitrogen per 100 gm of dry matter consumed. This is considerably lower than the values used by Blaxter and Mitchell. One would expect calves consuming entirely a liquid diet to excrete less metabolic nitrogen per unit of dry matter intake, since the fiber content of the diet determines to a large extent the ratio of the metabolic fecal nitrogen excretion to the dry matter consumed (Mitchell, '26).

It has been shown that the excretion of metabolic fecal nitrogen is influenced by body size as well as by the level of food intake. Schneider ('34) has reviewed the relative effect of these two factors. Table 3 illustrates the effect of body

size on the excretion of metabolic fecal nitrogen by cattle of different weights. The average value for the 4 calves reported on in this paper fits well with the general trend of the other data.

Blaxter and Wood ('51) reported a value of 0.43 gm of metabolic nitrogen per 100 gm of dry matter ingested by young calves consuming a liquid diet. This value was obtained, however, during the feeding of a nitrogen-free diet the digestibility of which was significantly lower than that of the normal diet. If, as suggested by these workers, metabolic nitrogen excretion is affected by the digestibility of the dry matter, the values determined under normal conditions

TABLE 4

Apparent and true digestibility of the nitrogen of casein

CATEGORY OF INTEREST	CALF NO.			
	1	2	13	14
Total N intake, gm per day	23.6	15.0	13.3	17.8
Total fecal N, gm per day	3.8	2.5	1.7	2.7
Metabolic fecal N, gm per day	2.9	1.1	0.9	1.4
N apparently digested, gm per day	19.8	12.5	11.6	15.1
N truly digested, gm per day	22.7	13.6	12.5	16.5
Apparent digestibility of N, %	84	83	87	85
True digestibility of N, %	96	91	94	93

of protein feeding should be more representative of the true metabolic nitrogen excretion, since digestibility of the dry matter of their nitrogen-free diet was considerably lower than that of the normal diet. Because the values reported in this paper were determined under conditions of normal intakes of protein as well as of total energy, it is the opinion of the authors that these values are more valid estimates of metabolic fecal nitrogen excretion than those obtained during the feeding of nitrogen-free or nitrogen-low diets.

In table 4 the apparent and true digestion coefficients of the nitrogen of casein are presented. These data indicate that the nitrogen of casein incorporated in purified liquid diets is readily digested by the calf.

SUMMARY

The metabolic fecal nitrogen excretion of 4 young dairy calves has been determined by feeding casein labeled with radioactive phosphorus, P^{32}, incorporated into a purified liquid diet.

The average metabolic nitrogen excretion was 0.27 gm per 100 gm dry matter intake. The validity of this value is discussed.

The true digestibility of the nitrogen of the casein used in these studies averaged 93.5%.

LITERATURE CITED

BLAXTER, K. L., AND H. H. MITCHELL 1948 The factorization of the protein requirements of ruminants and of the protein values of feeds, with particular reference to the significance of the metabolic fecal nitrogen. J. Animal Sci., *7:* 351.

BLAXTER, K. L., AND W. A. WOOD 1951 The nutrition of the young Ayrshire calf. I. The endogenous nitrogen and basal energy metabolism of the calf. Brit. J. Nutrition, *5:* 11.

BOSSHARDT, D. K., AND R. H. BARNES 1946 The determination of metabolic fecal nitrogen and protein digestibility. J. Nutrition, *31:* 13.

HARRIS, L. E., AND J. K. LOOSLI 1944 The minimum protein requirements of young Holstein calves. J. Dairy Sci., *27:* 650.

HARRIS, L. E., S. H. WORK AND L. A. HENKE 1942 The utilization of urea and soybean oil meal nitrogen by steers. J. Animal Sci., *2:* 328.

KAY, H. D. 1934 The chemistry and metabolism of the compounds of phosphorus. Ann. Rev. Biochem., *3:* 133.

KLEIBER, MAX, A. H. SMITH, N. P. RALSTON AND A. L. BLACK 1951 Radiophosphorus (P^{32}) as tracer for measuring endogenous phosphorus in cow's feces. J. Nutrition, *45:* 253.

LOFGREEN, G. P., MAX KLEIBER AND J. R. LUICK 1952 The metabolic fecal phosphorus excretion of the young calf. Ibid., *47:* 571.

LOFGREEN, G. P., MAX KLEIBER AND A. H. SMITH 1951 The digestion and absorption of P^{32} labeled casein by the young calf. Ibid., *43:* 401.

———— 1952 The excretion of injected P^{32} into the gastrointestinal tract of the young calf. Ibid., *47:* 561.

MITCHELL, H. H. 1926 The determination of the protein requirements of animals and of the protein values of farm feeds and rations. Bull. Natl. Research Council No. 55.

SCHNEIDER, B. H. 1934 The relationship of the metabolic nitrogen of the faeces to body weight and to food intake for rats. Biochem. J., *28:* 360.

———— 1935 The subdivision of the metabolic nitrogen in the feces of the rat, swine and man. J. Biol. Chem., *109:* 249.

SWANSON, E. W., AND H. A. HERMAN 1943 The nutritive value of Korean lespedeza proteins and the determination of biological values of proteins for growing dairy heifers. Missouri Agr. Expt. Sta. Research Bull., 372.